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Effect of Sodium Oleate on Growth of *Streptobacillus moniliformis*.* (18657)

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The effect of oleic and other fatty acids on the growth of various microorganisms is well known. The result may be one of stimulation, inhibition or bactericidal action depending upon the fatty acid and the concentration used. Kodicek and Worden(1) found that *Lactobacillus helveticus* was inhibited for 24 hours by oleic acid, and, for 72 hours or more by linoleic and linolenic acids when used in concentrations of 160 γ per 10 ml of medium. Dubos and Middlebrook(2) have shown that soaps of oleic and other fatty acids exert a marked bacteriostatic and bactericidal action on *Mycobacterium tuberculosis.* Wynne and Foster(4) have more recently reported that sodium salts of oleic and linoleic acids inhibited growth of *Micrococcus pyogenes var. aureus.* The inhibitory effect of oleic and other fatty acids can be neutralized by addition of certain substances such as albumin(2), starch(3), and vegetable lecithin (4) to the medium.

The present study was undertaken in order to determine the effect of sodium oleate on growth of *Streptobacillus moniliformis* in the

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2. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, v56, 334.

3. Ley, H. L., and Mueller, H., *J. Bact.*, 1946, v52, 4.

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presence of varying quantities of bovine serum and of animal lecithin.

Experimental. Three strains of *Streptobacillus moniliformis*, obtained from Dr. Fordyce R. Heilman, were used. Strains 124 and 1599 were obtained from cases of rat-bite fever. Strain 635 was isolated from a rat. The basal medium was tryptose phosphate broth adjusted to pH 7.6 to 7.8. Stock cultures of the 3 strains were maintained on this basal medium after addition of Seitz-filtered bovine serum in a final concentration of approximately 20%. The same basal medium was used but the concentration of Seitz-filtered bovine serum varied as indicated. In studying the effect of sodium oleate on growth of *Streptobacillus moniliformis*, the sodium oleate was dissolved in water and added to the basal medium. The medium was then tubed and autoclaved and sterile bovine serum was added just before inoculation. The animal lecithin (Nutritional Biochemicals Corp.) was dissolved in petroleum ether and added to each tube of the basal medium before autoclaving, the ether having been driven off in a water bath before the tubes were plugged. Sterile bovine serum was added to the medium before use.

Results. Since Wynne and Foster(4) reported that 90 γ of sodium oleate per ml of medium inhibited the growth of *Micrococcus pyogenes var. aureus*, this concentration was selected for use in a preliminary experiment. Tryptose phosphate broth containing 90 γ of sodium oleate per ml and approximately 20% of Seitz-filtered bovine serum was inoculated with 0.1 ml of a 1:50 dilution of a 24 hour stock culture of *Streptobacillus moniliformis*. No evidence of inhibition was observed. Experiments were then conducted to determine whether the toxic effect of sodium oleate could be demonstrated in the presence of smaller amounts of serum. Accordingly 0.25, 0.5 and 0.75 ml of Seitz-filtered bovine serum were added to 5 ml of tryptose phosphate broth containing 90 γ of sodium oleate per ml. These quantities of serum represent approximately 5, 10 and 15% concentrations. Controls containing the same amounts of serum, but no sodium oleate were included. The results of repeated experiments with the 3 strains are shown in Table I. The organism was completely inhibited in the presence of 5% serum while variable results were obtained in the medium containing 10% serum. Growth was consistently observed in the medium containing 15% serum.

TABLE I. Effect of Sodium Oleate, Serum and Animal Lecithin on the Growth of *Streptobacillus moniliformis.* 0.1 ml inoculum 1:50 dil. of 24-hr stock culture.

	Sodium oleate, 90 γ/ml				
	Serum			Lecithin*	
Strain	5%	10%	15%	100 γ/ml	200 γ/ml
124	—	±	+	—	—
635	—	±	+	—	—
1599	—	±	+	—	—

* Lecithin tested in the presence of 5% bovine serum.

— = No growth.
± = Variable results.
+ = Growth.

While 90 γ of sodium oleate in the presence of approximately 5% bovine serum consistently inhibited growth of the 3 strains such was not the case when the concentration of sodium oleate was reduced to 45 γ per ml. In this concentration of sodium oleate the results were irregular in that inhibition was observed on some occasions but not on others. These results suggest that the level of sodium oleate required to inhibit the growth of *Streptobacillus moniliformis* in the presence of 5% bovine serum is between 45 and 90 γ.

Because Wynne and Foster(4) reported that the inhibitory effect of 90 γ of sodium oleate per ml of medium for *Micrococcus pyogenes var. aureus* was neutralized by 100 γ of lecithin per ml of medium, experiments were conducted to determine whether the inhibitory effect of sodium oleate on the growth of *Streptobacillus moniliformis* could also be neutralized by lecithin. The results are shown in Table I. The following controls (not shown in the table) were included: (1) Basal medium containing oleate and serum and (2) Basal medium to which only serum was added. The results presented in Table I show that neither 100 γ nor 200 γ of lecithin per ml of medium neutralized the toxic effect of sodium oleate. The difference in results reported here as compared with those reported by Wynne and Foster(4) may well be due to the lecithin. Wynne and Foster employed vegetable lecithin, whereas in studies reported here animal lecithin was used exclusively.

Summary. Sodium oleate in a concentration of 90 γ of sodium oleate per ml of medium inhibits the growth of *Streptobacillus moniliformis.* This inhibitory effect was reversed by addition of adequate amounts of bovine serum. Animal lecithin in concentrations of 100 and 200 γ per ml of medium failed to neutralize the inhibitory effect of sodium oleate.

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Effect of Derivatives of L-Cysteine on the Growth of *Leuconostoc mesenteroides* P-60.* (18658)

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Sulfur containing substances play an important role in cellular metabolism. Some microorganisms require organic sulfur compounds for growth while others can utilize inorganic forms of sulfur(1-4). In a nutritional study of *Escherichia coli,* Bargoni(5) reported that alliin (S-allyl-L-cysteine sulfoxide), methionine, and cystine promoted growth of this organism. Work in this laboratory has shown that alliin also stimulates the growth of *Leuconostoc mesenteroides* but to a lesser degree than L-cystine. Since L-cystine is an essential amino acid for the growth of *Leuconostoc mesenteroides*(6,7), the stimulation observed for alliin may be exerted indirectly by participating in a biological synthesis of L-cystine. Because of the close relationship of alliin, L-cysteine and L-cystine and the possible participation of L-cysteine derivatives in the biological synthesis of L-cystine, it was of interest to determine the effect of various L-cysteine derivatives on the growth of *Leuconostoc mesenteroides.*

Experimental. Preparation of derivatives of L-cysteine. S-methyl-L-cysteine, S-ethyl-L-cysteine, S-allyl-L-cysteine, S-propyl-L-cysteine, S-ethyl-L-cysteine sulfoxide, S-propyl-L-cysteine sulfoxide, S-allyl-L-cysteine sulfoxide (alliin) and L-cystine disulfoxide were prepared by previously described methods of synthesis(8-11). All compounds were recrystallized from the appropriate solvent until acceptable nitrogen values and melting points were obtained. The properties of these compounds are summarized in Table I.

Method of assay. Leuconostoc mesenteroides P-60 was used as the test organism throughout the experiment. Riesen *et al.*(7) found that this organism responded to cystine in a consistent and reproducible manner. The derivatives of L-cysteine were assayed for

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TABLE I. Properties of L-Cysteine Derivatives.

Compound	Formula	m.p. °C (decomp.)	% nitrogen	
			Found	Calc'd
S-Methyl-L-cysteine	$CH_3SCH_2CH(NH_2)COOH$	240-243	10.6	10.4
S-Ethyl- ”	$C_2H_5SCH_2CH(NH_2)COOH$	234-236	9.4	9.4
S-Propyl- ”	$C_3H_7SCH_2CH(NH_2)COOH$	228-233	8.4	8.6
S-Allyl- ”	$C_3H_5SCH_2CH(NH_2)COOH$	214-216	8.7	8.7
S-Ethyl-L-cysteine sulfoxide	$C_2H_5SOCH_2CH(NH_2)COOH$	156-158	8.4	8.5
S-Propyl- ” ”	$C_3H_7SOCH_2CH(NH_2)COOH$	151-154	7.8	7.8
S-Allyl- ” ”	$C_3H_5SOCH_2CH(NH_2)COOH$	163-165	8.1	7.9
L-Cystine disulfoxide	$SOCH_2CH(NH_2)COOH$ | $SOCH_2CH(NH_2)COOH$	180-183	10.4	10.3
L-Cystine (control)	$SCH_2CH(NH_2)COOH$ | $SCH_2CH(NH_2)COOH$	254-259	11.8	11.7

TABLE II. Effect of Derivatives of L-Cysteine on Growth of *Leuconostoc mesenteroides* P-60.

Compound	ml of 0.05 N NaOH μg of compound per tube				
	0	5	10	15	20
S-Methyl-L-cysteine	1.02	1.02	0.98	1.01	1.03
S-Ethyl- ”	0.99	1.02	0.96	1.04	1.04
S-Propyl- ”	1.10	1.06	1.14	1.12	1.08
S-Allyl- ”	1.07	1.05	1.13	1.08	1.10
S-Ethyl-L-cysteine sulfoxide	1.05	1.05	1.12	1.08	1.14
S-Propyl- ” ”	1.12	1.41	1.42	1.65	1.98
S-Allyl- ” ”	1.06	2.15	3.05	4.12	4.84
L-Cystine disulfoxide	1.02	2.10	2.93	3.42	4.01
L-Cystine (control)	1.05	4.63	7.46	8.13	8.21

growth response by substituting them for L-cystine using the peroxide-treated peptone basal medium as described by Riesen *et al.*(7). The hydrogen peroxide-treated peptone was prepared according to the method described by Lyman *et al.*(12). Riesen *et al.*(7) found that from 44 to 48% of the cystine activity for *Leuconostoc mesenteroides* was lost by autoclaving the complete media in the usual manner. In view of this, the method employed successfully by Camien and Dunn(13) was used in assaying L-cystine and the L-cysteine derivatives. This method differs from the usual microbiological procedure in that sterile glucose solution is added aseptically to each tube of previously sterilized media containing all of the essential ingredients except glucose.

Results and discussion. From Table II, it is noted that only S-allyl-L-cysteine sulfoxide (alliin) and L-cystine disulfoxide stimulated the growth of *Leuconostoc mesenteroides* to any marked degree. Substitution of the sulfur hydrogen of L-cysteine by methyl, ethyl, allyl and propyl groups did not produce any observable response. A very slight but consistent growth response was noted in the case of S-propyl-L-cysteine sulfoxide. Since S-propyl-L-cysteine was found to have no activity, it would appear that the presence of the sulfoxide group in S-propyl-L-cysteine sulfoxide was responsible for this activity. It is interesting to note that S-ethyl-L-cysteine sulfoxide and S-propyl-L-cysteine did not promote growth of this organism while S-allyl-L-cysteine sulfoxide stimulated growth. This indicates that the activity of the latter compound may be due to both the presence of the sulfoxide group and the unsaturation in the allyl radical.

The possibility always exists that the activity of these various derivatives for *Leuconostoc mesenteroides* may have been modified by the use of the peroxide-treated peptone

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basal medium. Camien and Dunn(14) have found that the addition of oxidized casein hydrolysate to an otherwise synthetic basal medium, markedly suppressed the response of *Lactobacillus arabinosus* to D-methionine and DL-methionine sulfoxide.

Summary. 1. Alliin and L-cystine disulfoxide stimulated the growth of *Leuconostoc mesenteroides* but to a lesser degree than L-cystine. 2. Substitution of the sulfur hydrogen of L-cysteine by alkyl and alkene radicals did not produce any observable response. The sulfoxide of S-ethyl-L-cysteine was also inactive; however, S-propyl-L-cysteine sulfoxide was found to have a very slight activity. 3. The activity of alliin may be due to both the presence of the sulfoxide group and the unsaturation in the allyl radical.

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Incidence of Spontaneous Mammary Tumors in Mice with *Lithospermum*-Induced Diestrus.* (18659)

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Cranston, Kucera, and Bittner(1) reported that C_3H mice fed on a diet containing *Lithospermum* exhibited a decreased incidence of spontaneous mammary tumors: from the 58.1% norm to 2.8%. Setting out with the same endocrinological premises as the above workers, a program, begun by us in 1946, was likewise aimed at investigating the effect of *Lithospermum*-induced diestrus on the development of spontaneous mammary tumors in C_3H mice; and this program was in its concluding phases when the above-cited paper appeared. In view of potentialities in the pharmacological approach to tumor prophylaxis, we feel that our methods and results should be recorded for comparison with those of the Cranston group.

The rationale underlying this type of experimentation has been presented adequately in the paper by Cranston, *et al.* It suffices to say here that through the induction of sustained diestrus in C_3H mice by the administration of *Lithospermum* in the diet, it was hoped to impair the mammary tissue substrate on whose normal functioning the development of mammary carcinoma in C_3H mice is known to depend. We have reported earlier that the mammary tissue of *Lithospermum*-treated C_3H mice is rudimentary (2); and the endocrinic mechanism of *Lithospermum* action has recently been discussed by Drasher(3).

Procedure and results. About 400 virgin C_3H female mice† were divided into two groups: one control, the other experimental. All were 6 months old at the beginning of the experiment. Stainless steel cages, 11″ x 11″ x 6″, were used to house the mice; between 5 and 10 mice were maintained together in each cage. Coarse-wire hampers were kept filled with dietary pellets for *ad lib.* feeding. The control group was supplied with pellets of regular Rockland Mouse Diet; the experimental group with pellets of Rockland Diet containing *Lithospermum.* Methods for preparing such pellets have been described previously(2).

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* The work reported in this paper was supported in part by grants from the U. S. Public Health Service on recommendation of the National Advisory Cancer Council. Grateful acknowledgement is made to Dr. F. S. Cooper for advice as to presentation of the data in graphic form.

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† Purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

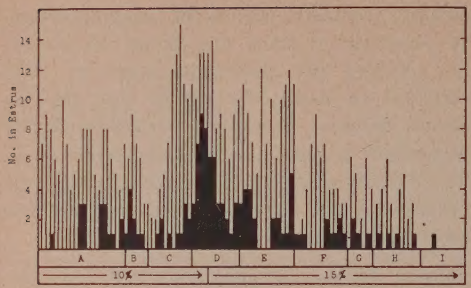

FIG. 1.

Number of mice in estrus for 97 samplings taken rotationally throughout both the control and *Lithospermum*-fed groups from the beginning of the experiment when the mice were six months old to the end of the experiment 18 months later. Each sampling comprised 25 mice. The samplings were not taken with absolute regularity throughout this period, rather Block A represents samplings taken during the 6-8 month period; B, 8-10; C, 10-12; D, 12-14; E, 14-16; F, 16-18; G, 18-20; H, 20-22; I, 22-24. Narrow lines, control mice; heavy lines, *Lithospermum*-fed mice. During the first two-fifths of the experiment the diet contained 10% *Lithospermum;* for the remainder of the experiment, 15% (see text for explanation).

At frequent intervals throughout the experimental period both the control and *Lithospermum*-fed groups were assayed for the state of their estrous condition, using standard lavage and smear methods(2). In order to obviate the simultaneous testing of all the nearly 400 mice, aliquots of 25 mice each were assayed throughout the experimental period. Such 25-mouse samplings were taken rotationally through both groups. The number of mice in each 25-mouse sampling showing estrus at the points of assay are presented in Fig. 1, with the data extending from the beginning of the experiment, when the mice were 6 months old and not yet in their normal tumor developing period, to the experiment's conclusion, about a year and a half later.

These data clearly indicate that while estrus was not wholly abolished in the *Lithospermum*-fed group, a very significant decrease in estrus frequency was sustained throughout the experimental period. Complete abolishment of estrus could easily have been achieved by merely increasing the *Lithospermum* content of the diet, but this was carefully avoided because, as known from earlier work(2), a high level of *Lithospermum* (say, 30%) would have resulted in an immediate loss in body weight. The level selected was that just great enough to induce considerable diestrus without on the other hand causing appreciable loss in body weight. This dosage level was determined to be between 10% and 15% by weight of powdered whole-plant *Lithospermum,* blended into Rockland Diet. Accordingly, the experimental group of mice was fed on a 10% *Lithospermum* diet for seven months; and then, as a slight refractoriness to the drug appeared to develop (Fig. 1), on a 15% diet for the remainder of the experiment.

That this dosage was appropriate in so far as growth was concerned is indicated from the data presented in Fig. 2. Here the body weight curves from both groups of mice, extending throughout the entire experimental period, indicate very little weight divergence from the norm on the part of the *Lithospermum*-fed group.

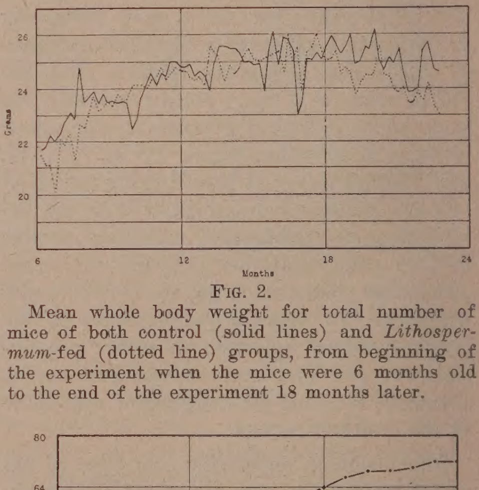

FIG. 2.

Mean whole body weight for total number of mice of both control (solid lines) and *Lithospermum*-fed (dotted line) groups, from beginning of the experiment when the mice were 6 months old to the end of the experiment 18 months later.

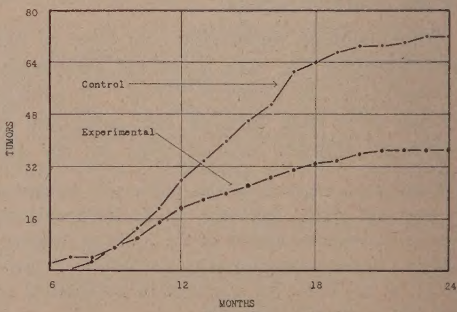

FIG. 3.

Cumulative number of tumors as of the beginning of each month throughout the experiment.

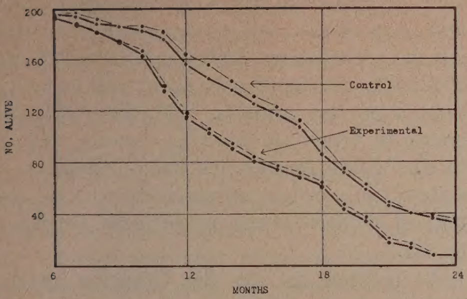

FIG. 4.

Total number of mice alive at the beginning of each month throughout the experiment. Points in heavy lines derived by subtracting deaths from all causes; points in lighter lines derived by subtracting deaths from causes other than tumors.

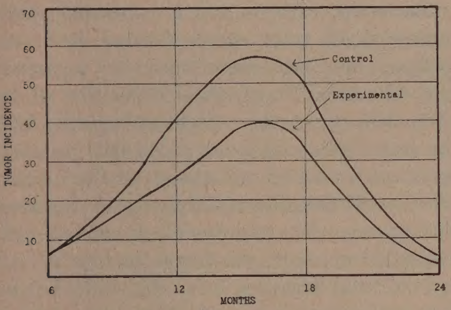

FIG. 5.

Tumor incidence, plotted as per thousand mice per month, throughout the experiment. The points from which these curves were drawn were derived from the experimental data as follows: Best fitting smooth curves were drawn by inspection through the points in Fig. 3 and 4. Points were read from these smooth curves and computed as $\triangle T/S$, where $\triangle T$ is the number of new tumors developing during each monthly period, and S is the number of mice alive at the beginning of each month. The resulting figures were plotted and the closest smooth-line curve fitted and drawn.

Fig. 3 depicts the cumulative incidence of mammary tumors from the beginning to the end of the experiment. At the end of the experiment about twice as many tumors had developed in the controls as in the *Lithospermum*-fed group: 36% in the former; 19% in the latter.

Survival curves for both control and experimental groups are presented in Fig. 4. Two sets of curves are given for each group: one showing the total number of deaths from all causes, the other showing deaths due to causes other than tumors. From a comparison of the two sets of curves it is seen that deaths among the *Lithospermum*-fed mice during the greater part of the experimental period occurred generally two to four months earlier than in the controls. Whether this was due to unidentified harmful effects of the *Lithospermum*, or to decreased resistance to infection, or to an acceleration of aging processes, cannot be said. It does mean, however, that the total number of *Lithospermum*-fed mice alive was always somewhat less than in the controls, and that the number subject to tumor development at any given time was consequently greater in the latter group. When the data are plotted as in Fig. 5—where differential monthly survival is taken into account—and the control and experimental curve areas measured and compared, the tumor incidence ratio for the two groups becomes 3 to 2.

Discussion. Two factors make it difficult to interpret the data presented here as well as those published by the Cranston group. First, there is the possible occurrence of inanition effects of the kind described by Tannenbaum(4) and by Huseby, Ball, and Visscher(5). By reducing the caloric intake these workers drastically lowered the incidence of spontaneous mouse tumors. Cranston, *et al.* showed that mice fed on a high *Lithospermum* diet secured less total nutrient than mice on normal diet; their *Lithospermum*-fed mice fell as much as 28% behind the gain in weight shown by the controls during the tumor developing period(1). In view of the work of Tannenbaum and of Huseby, *et al.*, this general growth retardation could account for the drop in tumor incidence in *Lithospermum*-fed mice reported by Cranston, *et al.*, a possibility which they themselves have pointed out. In our experiment, on the other hand, where the *Lithospermum* content of the diet was kept minimally low, and where the weight curves do not show much divergence (Fig. 2), it seems reasonable to assume that both experimental and control groups were nourished

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at substantially similar caloric levels, and that the factor of inanition was therefore not a major one in lessening the tumor incidence in the *Lithospermum*-fed mice.

A peculiarity in our data is the fact that only 38% of the controls developed tumors, whereas the strain of C_3H mice used by us has been reported as having as high as a 97% incidence of spontaneous mammary tumors(6). Our low control incidence is probably explicable on the grounds that keeping 5-10 mice in a cage constituted crowding and competitive feeding, factors which have been shown repeatedly to lower the incidence of tumors in this strain(7). On the other hand, these factors would not seem to have vitiated the experiment, for crowding was identical in both groups. The relative rather than the absolute tumor incidence in the control and *Lithospermum*-fed groups would seem to be the figures of significance.

As to the comparative death rates in the two groups, Cranston, *et al.* unfortunately do not give survival curves for their control and *Lithospermum*-fed mice, and hence their statement that the average non-tumor death age was 483 days for both groups cannot be fully appraised. Our own finding of an earlier susceptibility of non-tumor death in the *Lithospermum*-fed mice than in the controls, as plotted in Fig. 4, is a disturbing one and suggests the need for caution in interpreting the fact that the *Lithospermum*-fed group eventually developed only about half as many tumors as the controls. Whether this decrease in tumor incidence is directly related to the *Lithospermum*-fed mice being in a state of more-or-less sustained diestrus, we are not, on the basis of the present data, prepared to say.

As pointed out by the Cranston group, experiments of this sort could lead to more decisive results if pure extracts of the *Lithospermum* anti-estrous factor were available, thus minimizing possible side reactions of unknown identity.

Summary. About 400 6-month-old virgin female C_3H mice were divided into two groups: one was maintained on a normal diet; the other on an anti-estrous diet of 10-15% *Lithospermum.* Eighteen months later the latter group had developed half as many spontaneous mammary tumors as the former; however, if the differential mortality observed throughout the experiment is taken into statistical account, the tumor incidence ratio for the total period is 3 for the control, 2 for the experimental group. This reduction in tumor incidence was possibly connected with the *Lithospermum*-diestrus, but inanition and differential survival factors remain to be more fully evaluated.

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Neuropathology of Teschen Disease (Virus Encephalomyelitis of Swine).* (18660)

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(Introduced by Francis G. Blake)

From the 98th General Hospital, European Command, U. S. Army in Germany. Work done under auspices of Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, and the Section of Preventive Medicine, Yale University School of Medicine.

Teschen disease of swine is a virus encephalomyelitis which although its occurrence is unknown on the American continent, has been enzootic and epizootic in eastern Europe for at least 20 years. The disease is one which to date has been seen only in swine, and aside from the wild boar, no other susceptible host is known. It has been studied chiefly in Czechoslovakia where it has been most prevalent(1), and in Germany(2). Its virus etiology was demonstrated by Klobouk, who did the first and much of the subsequent experimental work with the disease(1,3). An extensive pathological study has been reported by Dobberstein(4), who mentioned the similarity between Teschen disease and poliomyelitis. Others(1,5,6) have also laid stress on this supposed similarity, and it is our purpose to consider the validity of the resemblance. In particular, we have attempted to determine whether the term poliomyelitis-like can be accurately applied to Teschen disease, and in this we have been guided by a recently published definition of poliomyelitis virus in which the differentiation is based in some measure on the distribution of lesions in the CNS(7). In the present study therefore, our interest was focussed on the type and distribution of CNS lesions in experimental Teschen disease, as compared with poliomyelitis and certain other neurotropic virus diseases.‡ At a later date a more detailed report including a comparison of the pattern of distribution of these lesions after various routes of inoculation, will be published.

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* Aided by a grant from the Associated Women of the American Farm Bureau Federation.

† Now with the Section of Preventive Medicine, Yale University School of Medicine.

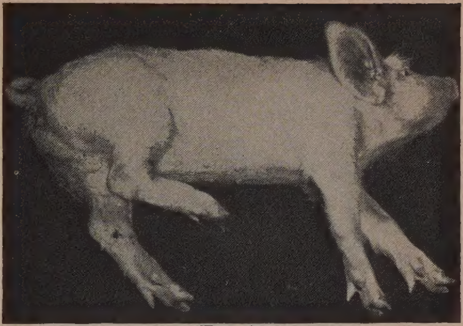

FIG. 1.
A pig showing the spasticity characteristic of the early stages of the disease. The animal was having almost continual convulsions.

The material studied consisted of brains and cords of 6 young pigs (*aet* 4-6 weeks) infected intracerebrally with 10% suspensions of several strains of virus.§ After an average incubation period of 6 days, the animals all showed the typical clinical course for this infection in severe form. The onset of the experimental disease was heralded by fever, followed, in order, by stiffness of the extremities (Fig. 1), ataxia, tremors, spastic convulsions, nystagmus, cranial nerve palsies, weakness or paralysis of the extremities and in the majority of cases, death. Not every animal showed all of these signs, and in the most severe cases coma, subnormal temperature and death supervened before the paralytic phase was clinically apparent. The duration of illness from onset to death or sacrifice thus varied between 24 hours and 4 days. At autopsy, the brain and cord were fixed in formalin, cut serially, and stained

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‡ We are greatly indebted to Professor W. Scholz, Director of the German Research Institute for Psychiatry, Munich, and Consultant in Neuropathology to the 98th General Hospital, for his continuous help and advice in this study.

§ We are indebted to the late Dr. Frantisek Gallia, National Institute of Health, Prague, for several strains of virus, and to Dr. Sven Gard, State Bacteriological Institute, Stockholm, and Dr. Pierre Lépine, Pasteur Institute, Paris, for kindly supplying us with their strains. The experimental work was all carried out in Germany because for obvious reasons it is not wise to import this virus into the United States.

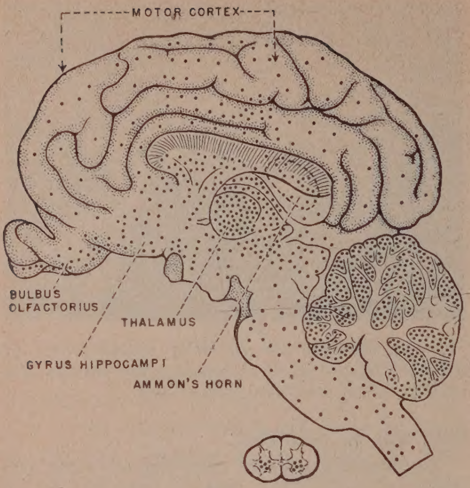

FIG. 2.
Diagram illustrating the distribution of lesions in the pig brain and medulla following intracerebral inoculation. (Projection of the lesions on the median surface of the brain).

with Nissl, or gallocyanin stains as indicated. Zenker fixed material was stained by the Giemsa method in a search for inclusion bodies.

On gross examination of the brain and cord, mild hyperemia was commonly noted, but edema was minimal even in the most severe cases. No abnormalities other than some congestion of the viscera were apparent in other organs. Histologically, the lesions were found to be virtually limited to the grey matter of the CNS. Perivascular cuffing and discrete nodules surrounding damaged neurons and blood vessels were characteristic. In some animals, rare perivascular cuffs were found in the white matter, and there was also occasional involvement of the undifferentiated nerve cells which are normally found in the matrix around the ventricles in very young animals such as those we used.

One of the most striking features of the pathological picture was the widespread distribution of cerebral and cerebellar lesions (Fig. 2). There was no part of the brain which was not at one time or another involved in the animals studied. However, the usual pattern was one in which the cerebellum was most heavily infiltrated, the Purkinje layer most severely, next the molecular, and least the granular layer (Fig. 3). At times there was disappearance of almost all the Purkinje cells over large areas. Next in density of lesions was the thalamus, then the basal nuclei (especially the striatum) and in general, the base of the brain. The olfactory apparatus which forms a relatively large part of the pig brain was severely involved: the olfactory bulbs, hippocampal gyrus, and Ammon's horn,—the entire rhinencephalon. The pons and the nuclei of the medulla showed usually a less severe reaction.

The motor cortex, which in the pig runs parallel to the median fissure and extends from the middle of the frontal lobe to the parietal lobe, was slightly more affected than the rest of the cortex, but the number of lesions here, although considerable, was far less than was found in the cerebellum or thalamus. It was characteristic for the massive involvement to be confined to the base and central parts of the brain, with a progressive diminution in the extent of lesions laterally and toward the periphery. The occipital lobes were almost spared.

In the spinal cord, the lesions were almost entirely in the anterior horns, with occasionally a few in the lateral and posterior horns (Fig. 4). In this respect the lesions resemble those of poliomyelitis and could be easily mistaken for them if examination of the brain were omitted. In Teschen disease, all levels of the cord tended to be equally affected. Two additional pigs killed 24 hours after onset of encephalitic signs, showed no cord lesions at any level, although the brain lesions were typical. Another animal, however, which had an unusually mild course clinically, showed very few lesions in the brain, but typical ones in the cord.

The meningeal reaction was usually mild, but occasionally more severe, and always most marked over the cerebellum. Lymphocytes and plasma cells composed the inflammatory exudate; polymorphonuclear leukocytes—as elsewhere—were strikingly rare. The choroid plexuses were similarly, but rarely affected.

Histologically, the primary attack of the virus appeared to be on the neurons. Although perivascular cuffing,—with lympho-

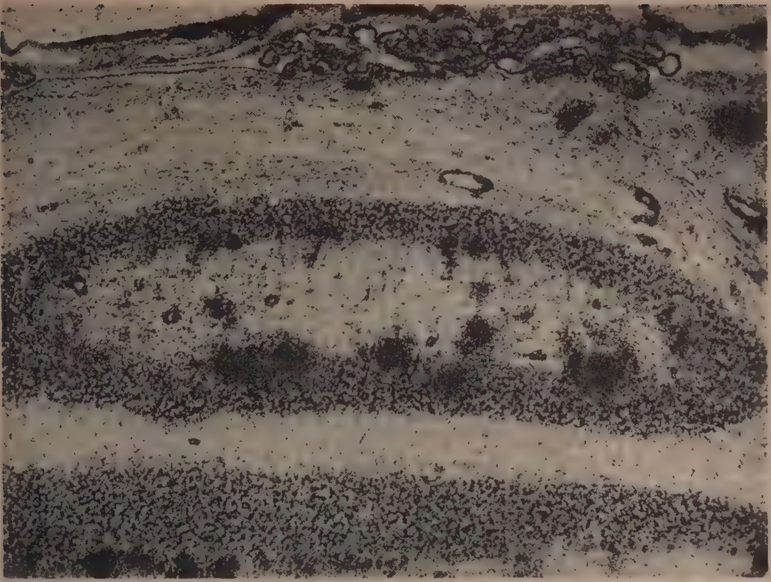

FIG. 3.

A section of the cerebellum underlying the choroid plexus of the 4th ventricle. Numerous nodules in the Purkinje layer are seen, and there is moderate perivascular cuffing. ×60.

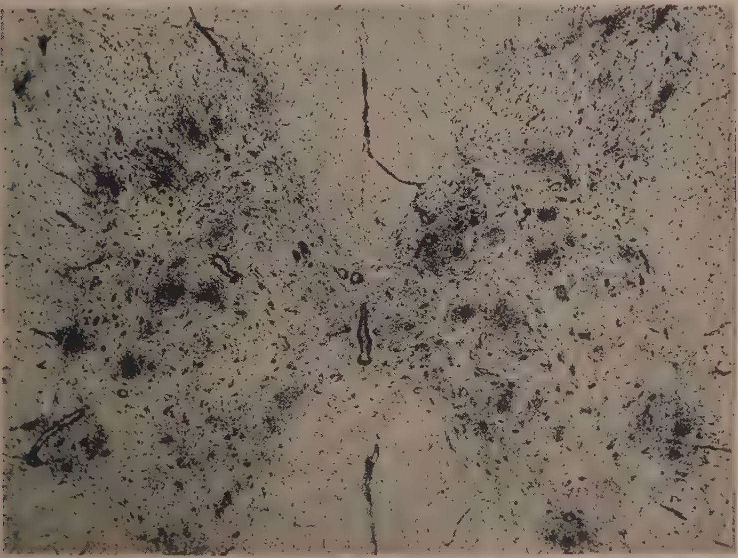

FIG. 4.

Section of the central area of the spinal cord, showing multiple neuronophagic nodules and perivascular cuffing. ×16.

cytes, polyblasts, and plasma cells—was characteristic, there was no evidence of necrosis of blood vessels. The damaged neurons showed first,—decrease in size of the Nissl bodies, then chromatolysis and disappearance of the Nissl substance, which often began at the periphery of the cell. Peripheral vacuolation sometimes accompanied this. Chromatolysis may progress to the point of complete dissolution of the Nissl substance so that the whole cell takes on a foggy reddish blue color with Nissl stain. Nuclear changes proceeded from pyknotic alterations in the chromatin, to karyorrhexis, karyolysis, and disappearance of the nuclear membrane, leaving a distorted and shriveled nucleus in a homogenized cytoplasm. Phagocytosis of dead cells seemed to be carried out chiefly by progressive forms of microglia; lymphocytes and occasional plasma cells were seen, but polymorphonuclear leukocytes were extremely rare in areas where phagocytosis was going on. Nerve cells may also undergo cytolysis, or vacuolation and absorption without neuronophagic reaction. Using Giesma stained sections, a thorough search for inclusion bodies was made, but none was found at any stage of neuronal damage.

The characteristic lesion in the brain and anterior horns of the cord was a collection of cells forming a nodule, which may either be found around a blood vessel, or around a damaged neuron. Nodules of varying size were composed of gliomesenchymal elements: micro and macroglia, lymphocytes, polyblasts and plasma cells. Here too, polymorphonuclear leukocytes were conspicuously absent.

In comparing the pathology of Teschen disease with other virus encephalomyelitides, it seems, Japanese B, and louping ill came particularly to mind for consideration. Although a similarity to poliomyelitis has been previously stressed, actually we were much struck by the differences, the most obvious being the widespread distribution of lesions in the brain in Teschen disease, in contrast to the highly selective distribution of lesions in the brain in poliomyelitis(8). It is true that certain areas of the brain and cord are severely affected in both diseases, but the pattern in Teschen—the extensive involvement of the cerebellum and basal nuclei, and the numerous lesions in all parts of the cortex—is completely unlike poliomyelitis in man or in the monkey. The similarity between the two diseases pathologically must rest on the spinal cord lesions. There is also some difference in the cellular reaction in that in Teschen disease, polymorphonuclear leukocytes are very rarely seen, while in acute lesions of poliomyelitis, they often form a part of the inflammatory reaction. But in an individual slide of a spinal cord it might be difficult to distinguish between the two. The lesions in Teschen disease in the pig brain and cord resemble strikingly those found in Japanese B encephalitis in man(9) both as to type and distribution. In fact, pathologically, Teschen disease is far more like Japanese B than like poliomyelitis. Widespread neuronophagic nodules and nodules associated with blood vessels are characteristic of both, but in Japanese B, the distribution of nodules tends to be more uniform throughout the brain, and the entire cerebral cortex is more densely involved, especially at the periphery, which is relatively spared in Teschen disease. There is also a more striking leptomeningitis in Japanese B, and although polymorphonuclear leukocytes are not prominent, they are not so rare as in Teschen disease. There are also close similarities between the pathological picture of Teschen disease in the pig and of louping ill(10), particularly experimental infections in the monkey(11), and in the pig(10,12).¶ The character and distribution of lesions in the cerebellum may be almost identical, although there tends to be a more pronounced cellular reaction in Teschen disease than in louping ill. The cortical and midbrain lesions in Teschen disease are more constant than in louping ill in the monkey,

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¶ We are grateful to Dr. J. Russell Greig, and Dr. D. R. Wilson of the Moredun Institute, Edinburgh, for making available to us sections and blocks of tissue from pigs which they infected with louping ill virus in 1930.

where, for instance the thalamus may be completely spared and the cortical lesions very scant(11).

Summary. A study of the neuropathology of Teschen disease reveals it to be a diffuse encephalomyelitis with characteristic lesions both as to type and distribution. The lesions are, with minor exceptions, confined to the grey matter of the brain and cord. Their widespread distribution in the CNS, and particularly their striking density in the cerebellum serve to distinguish Teschen disease from poliomyelitis, although spinal cord lesions may be similar in the 2 diseases.

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Methylation of Homocystine by Chicks Deficient in Vitamin B_{12}.* (18661)

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Gillis and Norris(1,2) and Schaefer, Salmon and Strength(3) have shown that chicks partially depleted of their reserves of vit B_{12} have an increased requirement for compounds which furnish labile methyl groups. Jukes, Stokstad and Broquist(4) have presented evidence which suggests that chicks deficient in vit B_{12} are unable to utilize homocystine plus betaine for the synthesis of methionine. Oginsky(5) concluded that the livers from vit B_{12}-deficient rats exhibit a lower ability to form methionine from homocystine and either choline or betaine as compared with livers from animals dosed with vit B_{12}. The work of Jukes *et al.*(4) and Oginsky(5) appeared to be in disagreement with the unpublished results of an earlier experiment conducted at this laboratory which showed that the methylation of homocystine by means of choline or betaine was not hampered by a deficiency of vit B_{12}. In view of this discrepancy the original study has been repeated and extended. The results of both experiments are presented in this report.

Experimental. The chicks used in this investigation were hatched from eggs laid by White Leghorn hens fed a diet containing no animal products or vit B_{12} and housed in pens with wire-mesh floors. The hatchability of the eggs was low, and the chicks were found in a preliminary experiment to be markedly responsive to vit B_{12} administration. The chicks were fed a vit B_{12} deficient diet containing 25% vegetable protein for a preexperimental period of 10 days. They were then uniformly distributed on the basis of weight among the experimental lots. Thereafter they were weighed daily at the same hour. The basal diet furnished approximately 0.2% methionine and was deficient in vit B_{12} and choline. In Exp. 1 it contained corn starch 65.55; isolated soybean protein 20; gelatin 4; L-cystine 0.15; soybean oil 2; ground cellophane 3; dicalcium phosphate 2; calcium carbonate 1; sodium chloride 0.5; dibasic potassium phosphate 0.5; magnesium sulfate 0.25; trace mineral mixture 0.05; and vitamin-starch mixture 1%. In Exp. 2 the basal diet was the same except that it contained 22% water extracted, isolated soybean protein and no gelatin, and hydrogenated vegetable fat was used in place of soybean oil. In Exp. 1 each lot contained 6 chicks and the experimental treatment lasted 5 days. In Exp. 2 each lot contained 5 chicks and the experimental period was of 9 days' duration.

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* Aided by a grant from the International Minerals and Chemical Corporation, Chicago, Ill.

† Research Associate.

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TABLE I. Growth of Vit. B_{12}-deficient Chicks Fed Various Supplements in a Diet Deficient in Methionine, Choline, and Vit. B_{12}.

Supplement, %	Avg wt gained 5-day period, g
0	—1.6
.3 choline Cl	3.7
.3 betaine HCl	0.9
.5 DL-homocystine	6.8
.5 DL-methionine	23.4
.5 DL-homocystine + .3 choline Cl	22.5
.5 DL-homocystine + .3 betaine HCl	23.2

TABLE II. Growth of Vit. B_{12}-deficient Chicks Fed Various Supplements With and Without Vit. B_{12}.

	Avg wt gained, 9 day period		
Supplement	B_{12}-deficient diet, g	3γ B_{12}/100 g diet, g	Increase due to B_{12}, g
0	6.6	23.6	17.0
.15% DL-homocystine	4.6	34.6	30.0
.15% DL-methionine	24.6	43.2	18.6
Homocystine + .172% betaine HCl	23.6	42.4	18.8
Homocystine + .156% choline Cl	31.4	53.4	22.0

The experimental plans are presented in Tables I and II respectively. To obtain a better conception of the relative efficiency of methionine and homocystine plus choline or betaine, the amounts of methionine and homocystine fed in Exp. 2 were reduced to 0.15% of the diet and the additions of choline chloride (0.156%) or betaine hydrochloride (0.172%) were made to correspond to the theoretical amounts necessary to methylate 0.15% homocystine.

Results. The chicks in Exp. 1 which received the unsupplemented basal diet failed to grow (Table I). Little or no effect was obtained by adding either choline or betaine alone, but the addition of homocystine alone resulted in a small improvement in growth. On the other hand when methionine, homocystine plus choline, or homocystine plus betaine were added growth improved significantly. Moreover, the results obtained with either of the two latter regimens were equally as good as those obtained with methionine. Vit B_{12}, therefore, did not appear to be directly concerned with the transfer of methyl groups from either choline or betaine to homocystine to form methionine. The small response obtained with homocystine alone indicates that methyl groups needed in this instance for the conversion of homocystine to methionine were obtained by synthesis.

In Exp. 2 the chicks which received the unsupplemented diet deficient in vit B_{12} grew very little and the addition of homocystine resulted in no improvement in growth (Table II). On the other hand, supplementing the deficient diet with either methionine, homocystine plus betaine, or homocystine plus choline significantly improved growth. The response due to supplementation with homocystine plus betaine was the same as that obtained with an equivalent amount of methionine, while supplementation with homocystine plus choline was slightly more effective, indicating that the choline deficiency was being partially overcome.

The basal diet with added vit B_{12} promoted significantly better growth than that obtained in the comparable lot fed the vit B_{12}-deficient diet. Adding homocystine alone to the vit B_{12}-adequate diet stimulated further growth. Since homocystine had no effect on growth in this experiment when added to the basal diet containing no vit B_{12}, this is regarded as evidence that in the chick, vit B_{12} is concerned in the utilization of homocystine in the absence of known methyl donors. Stekol and Weiss(6) have reported that rats are able to grow when fed a diet free of methionine and all known labile methyl donors, providing the diet contains vit B_{12} and homocystine. The results of the present work indicate that methyl synthesis also occurs at a suboptimal rate in chicks receiving vit B_{12}.

The effect of including vit B_{12} in the basal diet appears to have been strictly additive except where homocystine was supplied alone, and provides no evidence that the vitamin is required for the transfer of preformed methyl groups from either betaine or choline to homo-

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6. Stekol, J. A., and Weiss, K., *J. Biol. Chem.*, 1950, v186, 343.

cystine for the formation of methionine. The results obtained in this experiment, therefore, confirm the results obtained in Exp. 1.

Discussion. The results reported in this paper indicate that chicks severely depleted of their vit B_{12} reserves retain the ability to perform the over-all reaction: homocystine + labile methyl = methionine. If vit B_{12} is needed for the completion of this reaction, the amount required must be exceedingly small. It seems probable, however, in view of the results obtained with rats by Stekol and Weiss (6) and others, and the results presented here that vit B_{12} is concerned in the synthesis of methyl groups or the utilization of such synthesized groups by the chick.

The results presented here, showing that methionine formation occurred in vit B_{12}-deficient chicks supplied homocystine plus choline or betaine do not agree with those of Jukes *et al.*(4). This may be due to the fact that the diet used by the latter workers contained only 0.1% of betaine hydrochloride, an amount which on theoretical grounds could not methylate more than 0.087% of homocystine. The amount of homocystine furnished by these investigators was greatly in excess of this amount (0.6%). Furthermore, the basal diet used by Jukes *et al.*(4) contained 0.2% dimethylaminoethanol hydrochloride, a compound which presumably competes with homocystine for methyl groups. It appears, therefore, that the amount of methionine which could have been formed and made available for growth under these conditions must have been very small. This conclusion is supported by the fact that homocystine alone gave as good growth in the presence of vit B_{12} as did homocystine plus 0.1% betaine hydrochloride. There is thus no evidence that the efficiency of methyl transfer was increased by vit B_{12} in the work reported by Jukes *et al.*(4). Rather their evidence suggests a considerable synthesis of methyl groups in the presence of vit B_{12}.

Although Oginsky(5) reported that the livers of vit B_{12}-deficient rats exhibited a lower ability to form methionine from homocystine and either choline or betaine as compared with those from animals dosed with vit B_{12}, attempts by this investigator at *in vitro* activation of the methionine forming system by addition of solutions of crystalline vit B_{12} proved to be unsuccessful. Moreover it is clearly evident from Oginsky's(5) results that a very considerable synthesis of methionine occurred in the livers of the vit B_{12}-deficient rats, particularly in the presence of betaine.

Conclusions. Chicks very deficient in vit B_{12} grew as well when homocystine and betaine were added to a methionine-deficient diet as when a comparable amount of methionine was added. No evidence was obtained that vit B_{12} increased the efficiency with which preformed methyl groups were tranferred to homocystine since the effects of vit B_{12} were additive to those of methionine or its equivalents of homocystine plus labile methyl. It appears likely, however, that when the chick's diet lacks methyl groups vit B_{12} is concerned in the synthesis of such groups and their combination with homocystine to form methionine. Formation of methionine by this procedure, however, is limited and insufficient to meet the needs of the chick.

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Citrovorum Factor and Folic Acid in Treatment of Megaloblastic Anemia in Infancy.* (18662)

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Citrovorum factor is a nutrient required by *Leuconostoc citrovorum*(1). It is a metabolite of folic acid and is excreted in increased amounts in the urine of man and rats following the administration of folic acid(2). Its metabolic activity in man is indicated by its ability to reverse the toxic effects of aminopterin(3) and to induce a hemopoietic response in patients with pernicious anemia(4). Although the *in vitro* synthesis from folic acid and subsequent isolation in crystalline form has been accomplished(5), its structure has not been reported.

This report presents 2 patients with megaloblastic anemia of infancy who responded to this metabolite of folic acid and compares the effective dosage level of this metabolite with that of pteroylglutamic acid.

Observations. Wm. and Wa. G., white fraternal twins, were 10½ months old when admitted to Vanderbilt University Hospital in September, 1950. Their maternal grandfather had pernicious anemia. They weighed 3 lb. 5 oz. each at birth. Both infants were fed formulas of evaporated milk, later replaced by whole cow's milk. Solid foods had been offered but had not been taken in significant amounts. Orange juice and a multivitamin preparation had been given sporadically. Each twin had been admitted to the Nashville General Hospital on several occasions for acute infections. Three months before the present admission both had received transfusions of whole blood for a moderately severe hypochromic anemia.

Wm. had failed to gain weight and had an intermittent diarrhea and anorexia preceding his admission to the hospital. Examination revealed palor, irritability, generalized shotty lymphadenopathy and hepatosplenomegaly. The hemogram is shown in Fig. 1. Aspirated sternal marrow revealed an increased number of megaloblasts. He was placed on a diet of whole milk without other foods or vitamin supplements and given daily intramuscular injections of 500,000 units of citrovorum factor.‡ Seventy-two hours after the first injection there was a remarkable increase in the number of normoblasts in the marrow. On the 18th day the citrovorum factor was discontinued. His diet was supplemented with a multivitamin preparation which contained neither folic acid nor vit. B_{12}. He then received another course of treatment with twice as much citrovorum factor, but a recognizable secondary reticulocytosis did not occur. One month following discharge he was gaining weight and had an excellent appetite. The anemia has not recurred.

Wa. was admitted for study when the diagnosis in her twin was established. Physical examination revealed only pallor. Despite the relative mildness of her anemia, the marrow changes were more pronounced. She was also fed whole cow's milk exclusively and given 500,000 units of citrovorum factor intramuscularly daily. The hematologic changes are given in Fig. 2. On the 4th day of therapy the marrow specimen revealed active hemo-

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† Public Health Service Research Fellow of the National Institutes of Health.

‡ Supplied through the courtesy of Drs. T. H. Jukes and Harry P. Broquist of Lederle Laboratories.

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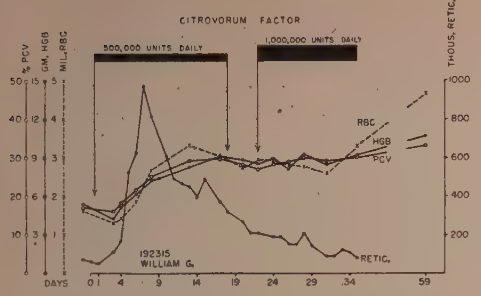

Fig. 1.

Case 1. Response of megaloblastic anemia in infancy to therapy with citrovorum factor.

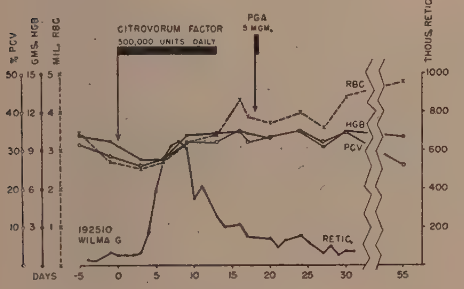

Fig. 2.

Case 2. Response of megaloblastic anemia in infancy to therapy with citrovorum factor. Failure to obtain an additional response to folic acid.

poiesis with normoblasts predominating. After 13 days this therapy was discontinued and she was started on a multivitamin preparation and given a single intramuscular dose of 5 mg of folic acid. No secondary reticulocytosis occurred. Following discharge from the hospital she continued to thrive.

Each of these twins received 500,000 units of citrovorum factor daily parenterally during the initial period of treatment. This is equivalent to 75 μg of the anhydrous free acid. Doubling the amount of this factor in the first case and giving a large dose of folic acid in the second produced no further response. Therefore, we may assume that the minimal effective dose of citrovorum factor is less than 75 μg daily. The minimal effective dose of folic acid has not been determined. However, it has become apparent that much smaller doses than the usually recommended 5 to 15 mg daily may produce a remission.

It is of interest, therefore, to consider 2 cases of megaloblastic anemia of infancy which we have treated with relatively small doses of pteroylglutamic acid. Both of these patients were comparable to the cases reported above in detail. The first of these patients (Fig. 3) failed to respond to the parenteral administration of 30 μg of vit. B_{12}, was subsequently given a small transfusion, and later treated with the daily oral administration of 500 μg of folic acid. A reticulocytosis, increase in the red cell count and hemoglobin concentration, and reversion of the bone marrow to normal followed. In the final patient (Fig. 4) the oral dose of folic acid was further reduced to 200 μg daily. Despite the absence of prolonged observations on this infant, it is apparent that a good initial response occurred. This was accompanied by a change in the marrow from megalo-

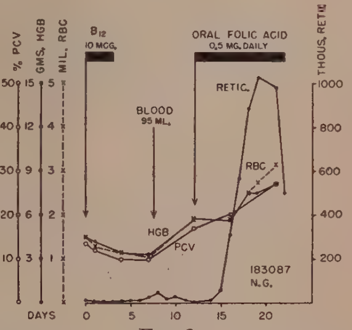

Fig. 3.

Hemogram of a patient with megaloblastic anemia in infancy who failed to respond to vit. B_{12} and later responded to 500 μg of folic acid by mouth daily.

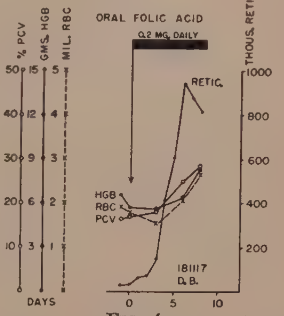

Fig. 4.

Hemogram of a patient with megaloblastic anemia in infancy who responded to the oral administration of 200 μg of folic acid daily.

blastic to normoblastic hyperplasia.

Discussion. It is of considerable interest that this metabolite (citrovorum factor) of pteroylglutamic acid proves active in the treatment of megaloblastic anemia of infancy. Our results suggest that the minimal effective quantity of citrovorum factor administered parenterally is less than 75 μg daily. Since the minimal effective daily dose of folic acid is obviously less than 200 μg orally, it is not yet possible to decide whether one of these agents possesses greater activity than the other. The pathogenesis of megaloblastic anemia is not established. The dietary need for folic acid by the healthy infant has not been demonstrated. The further study of the minimal effective dose of the hemapoietic agents related to folic acid should add considerably to our knowledge of the maximal dietary requirement, if any, for these factors.

From investigations of the experimentally induced megaloblastic anemia in the young monkey, May and his co-workers(6) have suggested that the scorbutic monkey may be unable to metabolize effectively folic acid to citrovorum factor. This hypothesis is consistent with the observation of Nichol and Welch(7) that the production by liver slices of citrovorum factor from pteroylglutamic acid was enhanced by ascorbic acid. It is important to recognize that none of the patients included in this report were scorbutic and that biochemical assessment of their nutriture for vit. C indicated that it ranged from moderately good to poor.

Summary. 1. Citrovorum factor in parenterally administered doses of 75 μg daily produced a remission in 2 patients with megaloblastic anemia of infancy. 2. Folic acid was effective in 2 cases of this syndrome at oral dose levels of 500 μg and 200 μg daily.

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Vitamin B_{12} Sparing Action of Aureomycin in the Rat.* (18663)

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Studies in this laboratory designed to elucidate the mechanism of action of folic acid antagonists have dealt with the so-called "folic acid" deficiency induced by aminopterin and that induced by including sulfaphthaladine in the diet of rats. It was found by Waisman *et al.*(1) that aureomycin could counteract the aminopterin and produce growth superior to the basal diet alone. During these studies it was necessary to test the relationship of aminopterin and aureomycin to vit. B_{12}. It was found that aminopterin exerted its antagonism to folic acid (PGA) with or without vit. B_{12}, and that aureomycin could completely replace vit. B_{12} in the diet of rats.

Experimental. Twenty-four day old weanling rats of the Sprague-Dawley strain, housed in individual screen cages, were fed a synthetic diet consisting of sucrose 73 parts; salt mixture,† 4 parts; thiamine, 0.5 mg; riboflavin, 0.5 mg; niacin, 1 mg; calcium pantothenate, 2 mg; pyridoxine, 0.5 mg; inositol, 100 mg; biotin, 0.002 mg; and choline, 300 mg. Two drops of oleum percomorphum were given by mouth twice weekly to each rat as a source of vit. A and D. Vit. B_{12} was added at a 0.010 mg/100 g level. Crystalline aureomycin was included in the diet at levels of 5 and 10 mg per 100 g.

Results and comment. Fig. 1 shows a typical experiment in which aureomycin was able

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* Supported in part by a grant from the American Red Cross (Atomic Energy Commission).

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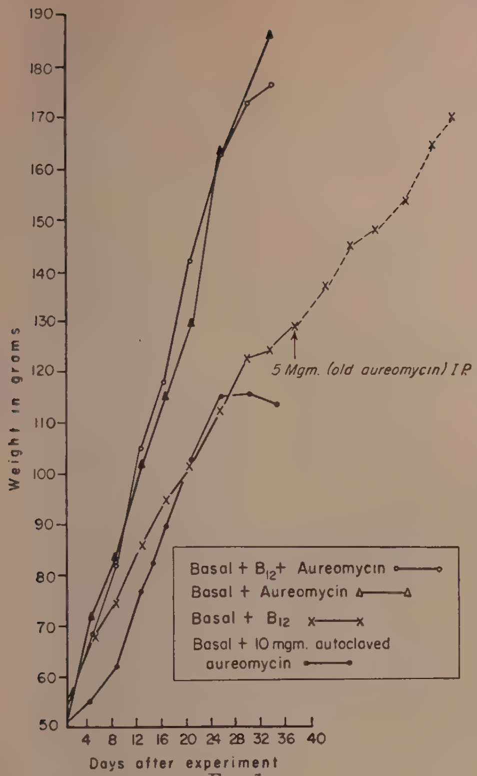

FIG. 1.

Effects of aureomycin on vit. B_{12} sparing action in the rat.

to give growth increases equal to or better than vit. B_{12}. It will be seen that the addition of aureomycin to the basal diet gives as good growth as basal plus vit. B_{12} plus aureomycin. The figure also indicates that "old aureomycin" given intraperitoneally (I.P.) was unable to provide any change in growth rate after the weight plateau was reached in the basal group. This preparation of intravenous aureomycin made up 2 months prior to its use has little or no antibiotic activity and thus provides further proof that the antibiotic potency of aureomycin is essential for the growth promoting effect. These results have been verified by repeated series in which a total of 50 animals was used.

The work of Oleson *et al.*(2) showed that chicks were able to grow better with aureomycin only if adequate amounts of vit. B_{12} were included. They showed further that rats grew slightly better (5 g/week) on the yellow corn-soybean meal diet with 10 mg aureomycin than without aureomycin. Stokstad and Jukes(3) also showed that aureomycin gave better growth responses in chicks when vit. B_{12} was included in the corn-soybean-alfalfa diet. Both groups of Lederle workers were able to obtain these results on a natural diet, but it is of interest that the "vit. B_{12} sparing" action of aureomycin was demonstrated in our rats fed a purified synthetic diet. It may be that with a purified diet the antibiotic is able to favor those bacteria which can synthesize vit. B_{12} required by the rat. If aureomycin decreases the number of *E. coli* in the intestinal tract of the rat and allows the increase of other bacteria such as *Bacillus megatherium*, which can produce adequate quantities of vit. B_{12}(4), then optimum growth responses can be expected. The weight of evidence would favor the hypothesis that aureomycin suppresses certain microorganisms that utilize vit. B_{12} for their growth and make it unavailable to the rat, or that the suppression of some microorganisms allows others to flourish, thus providing increased synthesis of vit. B_{12}.

Summary. Aureomycin can replace vit. B_{12} in the purified diet of the rat. A possible explanation for these findings must consider the influence of the antibiotic on intestinal synthesis in the rat. When the antibiotic activity is destroyed, the growth response is not observed.

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Effect of ACTH on Blood Glutathione and Sulfhydryl Content of Liver and Kidney in Rats.* (18664)

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(Introduced by Maxwell Finland)

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The effects of the 11, 17 oxysteroids of the adrenal cortex on carbohydrate metabolism have long been recognized(1). ACTH may induce glycosuria and hyperglycemia in some human patients and such disturbances in the metabolism of carbohydrate have been accompanied by a lowered blood glutathione (GSH)(2,3).

It has been suggested that some of the effects of the adrenal cortical hormones may be explained on the basis of interference with transphosphorylation enzyme systems(4,5). Sulfhydryl (SH) groups have been shown to be essential for a variety of enzymic activities, including many enzyme systems concerned with anaerobic glycolysis and phosphorylation(6,7). Therefore, the effect of ACTH on the SH contents of rat blood, liver and kidneys was investigated and the effect of the hormone on the ascorbic acid contents of the organs was also determined.

Methods. Twenty-four white male rats of the Sprague-Dawley strain, each weighing between 160 and 190 g, were allowed to feed *ad lib.* on a standard pellet diet. Six animals were given 3 mg of ACTH‡ (Armour standard LA-1A) subcutaneously every 8 hours for 3 days, and 6 animals received 3 mg of ACTH every 8 hours for 10 days. Prior experiments had shown that this schedule of dosage produced sustained eosinopenia in the rats. The remaining 12 animals served as controls, but in order to avoid nonspecific adrenal activation associated with injection alone they were not given control injections(8). Following a fast of 4-10 hours, each animal was anesthetized with ether. Three ml of blood were collected in potassium and ammonium oxalate following section of the tail. Portions of the right lobe of the liver and decapsulated kidney, each about 500 mg in size, were quickly removed from the anesthetized animal and weighed to within .05 mg on a torsion balance. The tissues were then placed in Potter-Elvehjem homogenizing tubes containing ice-cold 0.85% saline which had previously been boiled. The final volume was brought to 5.0 ml, and the tissues were then homogenized. The ascorbic acid contents of the homogenates were determined by the macromethod of Mindlin and Butler(9), substituting 1.0 ml of homogenate for 1.0 ml of plasma. The non-protein SH was measured by the amperometric method described by Kolthoff and Harris for mercaptans(10), adapted for use with amino acids and proteins by Benesch and Benesch (11), and further modified by Weissman, Schoenbach, and Armistead(12). Two ml of

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* Aided by a grant from U. S. Public Health Service.

† Senior Fellow in Virus Diseases, National Research Council.

‡ Kindly supplied by Dr. John R. Mote of Armour & Co.

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the homogenate were diluted with 16 ml of boiled distilled water and 2.0 ml of 22% sulfosalicylic acid were then added. Five ml aliquots of the protein-free filtrate were brought to a volume of 50 ml with 90% methanol, and the SH contents were titrated in duplicate with .001 N silver nitrate. The results were expressed as mg of glutathione. The addition of several drops of a solution of sodium ethylene diamine tetraacetate (EDTA)[§] stabilized both the SH and ascorbic acid contents of the filtrates by chelating any heavy metal ions present. Several more drops of EDTA were added to the filtrate-methanol mixture prior to titration. The total SH was determined by the method of Hughes(13). Methylmercury iodide in toluene was equilibrated with aliquots of the homogenate at pH 8.0, and the remaining methylmercury iodide titrated in duplicate with diphenylthiocarbazone. "Protein SH" was determined by subtracting the SH equivalent of the nonprotein SH from the total SH. Nitrogen concentrations of the homogenates were determined by the method of Johnson(14). The blood GSH was measured by the method of Woodward and Fry(15), and the blood sugar determined by the method of Reinecke(16).

Results. The findings in the blood are shown in Table I and those in liver and kidney are given in Table II. The blood sugar values in the rats receiving ACTH, under the conditions of fasting and anesthesia, were moderately elevated as compared with the controls. None, however, were in the diabetic range. Random urine samples showed only an occasional trace of sugar, and none of the animals was glycosuric following the short fast on the day it was sacrificed. No change in the blood GSH occurred under the influence of ACTH, nor were the erythrocyte counts significantly altered. The GSH content per million erythrocytes was in the normal range for rats(17).

TABLE I. Effect of ACTH on Blood Glutathione (GSH), Sugar and Erythrocyte Count of Rats.

ACTH given*	Blood GSH per million RBC, µg × 10^2	RBC, × 10^6/cu. mm	Blood sugar, mg/100 ml
Control	7.9 ± 1.2[†]	5.8 ± 0.8	109 ± 12
3 days	7.4 ± 1.5	6.7 ± 1.3	142 ± 18
10 days	7.6 ± 1.0	6.1 ± 0.9	138 ± 8

* 3 mg every 8 hr, subcutaneously.

[†] Values are expressed as the means ± standard deviation.

The mean nonprotein SH concentration in the kidney decreased within 3 days, but was not reduced further at the end of 10 days. These changes were significant statistically. There was no change in the mean nonprotein SH concentration in the liver after 3 days, but it was significantly increased after 10 days of administration of ACTH.

The mean concentration of ascorbic acid in the kidney was reduced in those rats given ACTH for 3 days, but showed no further change at the end of 10 days of therapy. No significant changes occurred in the hepatic concentration of ascorbic acid in either group receiving ACTH.

The "protein-SH" concentration per mg of nitrogen in either kidney or liver did not change significantly during the administration of ACTH.

ACTH apparently did not induce alterations in the hydration of the tissues as measured either by total nitrogen determinations or by erythrocyte counts.

Discussion. The blood GSH of human patients receiving ACTH has been reported to decrease in those individuals manifesting hyperglycemia or glycosuria(2,3). In these experiments, the blood GSH of the rats receiving ACTH was unchanged. The alterations in tissue nonprotein SH suggest that the measurement of GSH in the blood need not reflect the status of nonprotein SH compounds in the tissues. A dissociation between the blood and

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[§] "Sequestrene," kindly supplied by Alrose Chemical Co., Providence, R. I. (See Ref. 11).

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TABLE II. Effect of ACTH on Nitrogen, Sulfhydryl, and Ascorbic Acid Contents of Livers and Kidneys of Rats.

ACTH given*	Nonprotein SH, mg GSH/100 g	Total N, mg/100 mg	Protein SH, mg/100 mg N	Ascorbic acid, mg/100 g
		Liver		
Control	195 ± 14.6†	2.70 ± .36	1.43 ± .35	24.4 ± 1.7
3 days	196 ± 21.6	—	—	21.8 ± 3.9
10 days	228 ± 11.6‡	2.60 ± .12	1.36 ± .33	24.1 ± 3.0
		Kidney		
Control	166 ± 7.6	2.77 ± .42	1.05 ± .32	22.1 ± 2.1
3 days	129 ± 12.0‡	—	—	13.0 ± 2.4‡
10 days	119 ± 5.1‡	2.80 ± .29	1.29 ± .44	15.0 ± 2.1‡

* 3 mg every 8 hr, subcutaneously.

† Values are expressed as the means ± stand. dev.

‡ Differences from controls are statistically significant ($P < 0.01$ by "t" test of Fisher) (30).

tissue concentrations of GSH has also been demonstrated in scurvy(18). The values obtained for the hepatic nonprotein SH by the amperometric method agree quite well with hepatic GSH values obtained by the specific manometric method(19), indicating the GSH is the principal acid-soluble nonprotein SH compound in the liver. This correlates well with observations that the livers of rats contain enzymes capable of synthesizing but not hydrolyzing GSH(20). The increase in the hepatic nonprotein SH may therefore be explained by specific activation of enzymic synthesis of GSH in the liver, or by an increase in the availability to the liver of the constituent amino acids of glutathione, resulting from either increased hydrolysis of GSH in the kidney or from a catabolic phase of protein metabolism.

The renal nonprotein SH concentration as determined amperometrically was higher than that reported with the manometric method of GSH determination(19). The presence of a GSH-hydrolyzing enzyme in the rat kidney(20) may explain the occurrence in the kidney of large amounts of acid-soluble SH compounds other than GSH, and the changes induced by ACTH may represent loss of GSH or other SH compounds, or both. The decrease in the nonprotein SH of the kidneys of rats receiving ACTH is of interest in view of the evidence that altered renal tubular function occurs in human beings given large doses of ACTH or cortisone(21). The marked effects of various agents which inactivate sulfhydryl groups on the function and structure of the renal tubules are well known(22,23). It is of interest that the content of protein SH in neither kidney nor liver was altered by ACTH, despite the occurrence of changes in the nonprotein SH. Whether, as has been suggested(24), a diminution in GSH alone can alter in the kidney or elsewhere the activity of enzymes which depend on intact sulfhydryl groups remains to be determined.

Although the blood sugar values of the treated animals were higher than those of the controls, the possible hyperglycemic effect of the ether anesthesia(25) prevents precise interpretation. The hyperglycemia of the rats receiving ACTH may represent an increased responsiveness of these rats to anesthesia as a result of the increased glycogen content of the livers(26).

No explanation is apparent for the striking

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decrease in the renal concentration of ascorbic acid under the influence of ACTH. Increased urinary excretion of ascorbic acid, possibly as a result of a change in renal function, has been reported in human beings receiving ACTH(27). The administration of doses of cortisone adequate to suppress the growth of granulation tissue does not significantly alter the concentration of ascorbic acid in the plasma or muscles of rabbits(28). There is evidence suggesting that GSH serves to maintain ascorbic acid in the reduced state *in vitro*, and the amounts of ascorbic acid and GSH in the tissues may vary together under certain circumstances(18,29). Whether the reduction in renal nonprotein SH observed in this experiment had any causal relationship to the concomitant decrease in the concentration of ascorbic acid is not clear at this time.

Summary. Rats given ACTH for 3 to 10 days in doses sufficient to produce sustained eosinopenia when compared with controls, showed: (1) no change in the blood glutathione; (2) decreased nonprotein sulfhydryl contents of the kidneys; (3) increased nonprotein SH contents of the livers; (4) markedly decreased renal ascorbic acid; (5) no change in the ascorbic acid contents of the livers; and (6) no change in the protein sulfhydryl contents of the livers or kidneys.

The authors are indebted to Dr. Emanuel B. Schoenbach for advice concerning the use of the amperometric technic and to Miss Helen Alpert and Miss Marguerite M. Lundgren for technical assistance.

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Propagation of Poliomyelitis Virus in Cultures of Monkey and Human Testicular Tissues.*† (18665)

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It was recently established that propagation in tissue culture of members of the poliomyelitis group of viruses can be affected in extraneural tissues(2-5) as well as in neural tissues(1-4). Human embryonic brain and cord were employed for the cultivation of the MV(1), Lansing(2-4), and Brunhilde(3) strains; a mixture of human embryonic skin, muscle and connective tissue for the Lansing(2-4) and Brunhilde(3) strains; human foreskin and subcutaneous tissues from patients ranging in age from 4 to 11 years for the Lansing strain(3); human embryonic intestine for the Lansing strain(2,4); human adult testicular tissue for the Lansing and Hof.

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† Presented before the Am. Assn. of Immunologists, Federation of Am. Societies for Exp. Biol., April, 1951.

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strains(5). Dr. Evans kindly made known the results of this latter study before its publication(5). The importance of this discovery was obvious both from the practical point of view because of the ready availability of human testicular tissue, since patients commonly are subjected to castration for its hormonal palliative effect on inoperable carcinoma of the prostate, and because of the theoretical implication of this evidence that poliomyelitis virus can propagate in tissues devoid of neural elements. Accordingly, the efforts that had been currently under way in this laboratory to establish poliomyelitis virus in tissue culture were altered in an attempt to confirm the observations of Smith, Chambers, and Evans(5), and to extend their findings by employing the monkey as an even more readily available source of testicular tissue. Accordingly, 4 strains of poliomyelitis virus (murine-adapted Yale-SK, murine-adapted Lansing, Mahoney, and Minnesota, 1949) are under study in tissue cultures by employing for the maintenance of monkey testicular cells a technic described earlier(6). It is the purpose of the present communication to make known the evidence that establishes conclusively the propagation of poliomyelitis virus, Y-SK and Lansing strains, in monkey testicular tissues *in vitro*.

Materials and methods. Virus. The 2 strains of murine-adapted human poliomyelitis virus, Lansing and Yale-SK, were obtained from Dr. Joseph Melnick. The inoculum employed to initiate each experimental series in tissue culture was the supernatant fluid following centrifugation at 2500 r.p.m. of a mixture of mouse brain and cord in 10% suspension. At that time, the LD_{50} of the Lansing strain was $10^{-3.33}$ and that of the Y-SK strain was $10^{-3.1}$. *Tissue cells.* Testicular tissue, as whole testicle contained in its tunica vaginalis, was provided by castration of man or monkey. The human testicles were obtained from elderly men.§ Monkey testicles were obtained from fully mature *Macacus cynomolgus*, except for the first and third replacements, when rhesus monkeys provided the tissues. A newly removed testicle was employed for each tissue transfer. *Technic for tissue culture.* The technic employed was essentially similar to that utilized for the cultivation of the Minnesota strain of Coxsackie virus(6). Four flasks as a unit were made ready by inserting on the bottom of each a circular sheet of Beckley, OC design, perforated cellophane.||

Small fragments of testicular tissue were excised, minced finely to a maximal diameter of from 1 to 1.5 mm, and from 6 to 10 pieces were introduced beneath the cellophane membrane of 3 of the 4 flasks. The fourth flask in each unit, by receiving all material except tissue fragments, served as a control to test for the survival of virus in the absence of cells. Successive steps thereafter consisted of the introduction into each of the four flasks of 0.1 ml of the test virus suspension, 5 ml of nutrient fluid consisting of one part Simms' Ox Serum Ultrafiltrate¶ and two parts Simms' 3-X6, or 3-X7 salt solution, 0.1 ml sodium or potassium penicillin (50,000 units per ml) and 0.1 ml of streptomycin or di-hydrostreptomycin (50 mg per ml). Incubation was carried out at 36°C. On the third and sixth day thereafter, the maximal amount of overlying fluid was removed from above the cellophane membrane and replaced by 5 ml of fresh nutrient fluid; on the ninth day, the supernatant fluid from each of the three flasks containing tissue was pooled, and 0.1 ml was removed for transfer to each of the 3 test flasks containing fresh testicular tissue. This routine was maintained throughout except for Series C in which both human and monkey testicular tissues were employed in the propagation of Yale-SK virus. In this series, human testicular tissue was employed to initiate the series, but when it was not available after 12 days of culture, fresh monkey testicular tissue was used thereafter. A second exception in the routine procedure occurred on the sixth tissue passage after 6 days in culture when the replacement

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§ Testicular tissue was kindly supplied by Dr. C. D. Creevy, University Hospitals, Minneapolis, and by Drs. T. H. Sweetser and W. H. Card, Minneapolis General Hospital.

|| As approved by the Tissue Culture Commission.

¶ Purchased from Microbiological Associates, Coral Gables, Fla.

TABLE I. Results of the Propagation of the Lansing Strain in Tissue Culture, Series A.

GROWTH OF POLIOMYELITIS VIRUS (LANSING)
IN CULTURES OF TESTICULAR TISSUE
FROM MONKEY

FLUID FROM	RESULTS IN MICE	LOG OF DILUTION OF ORIGINAL VIRUS BY FLUID REPLACEMENT							
		2.7 3.6 4.5	6.2 7.1 8.0	9.7 10.6 11.5	13.2 14.1 15.0	16.7 17.6 18.5	20.2 21.1 22.0	23.7 24.6 25.5	27.2 28.1 29.0
TISSUE FLASKS	PARALYSIS†	3/6*	1/6	1/8 2/6 2/6	1/6 1/6 3/6	0/6 2/6 2/6	1/6 0/6 4/6	0/6 2/6 3/6	1/6 2/6 1/6
	DEATH†	4/6	3/6	2/8 2/6 6/6	3/6 5/6 5/6	2/6 4/6 6/6	4/6 4/6 5/6	2/6 6/6 6/6	2/6 3/6 1/6
CONTROL FLASKS	PARALYSIS	0/5	0/6		0/5				
	DEATH	0/5	0/6		0/5				
		2.7	4.4	6.1	7.8	9.5	11.2	12.9	14.6
		LOG OF DILUTION OF ORIGINAL VIRUS BY TISSUE REPLACEMENT							

* Denominator signifies No. of mice inj. intracer., .03 ml. Numerator signifies No. that died.

† "Paralysis" signifies No. of mice paralyzed (one observation per 24 hr). Paralysis within 24 hr of inoculation was not included. "Death" signifies No. of mice dead, excluding those dying within 48 hr of inoculation.

of monkey testicular tissue was effected, instead of observing the customary 9-day interval between the replacement of tissue fragments. The utilization of the especially designed tissue culture flask and of Mohr clamps for closure made it possible to pass daily over the content of each flask a gaseous mixture made up either of 5% carbon dioxide, 21% oxygen and 74% nitrogen, or of 5% carbon dioxide and 95% air. The control series of flasks was treated similarly, but independently. This control series of flasks was employed to learn the survival of virus in the absence of tissue. A second control, which was irregularly employed, consisted of a flask containing tissue, nutrient fluid, penicillin and streptomycin, but no virus.

Measurement of results. The presence of virus in tissue cultures was determined routinely in 15- to 25-day-old Swiss albino mice, CFW strain, by the injection intracerebrally, 0.03 ml, of undiluted, or successive 10-fold dilutions of pooled supernatant fluid which had been harvested from the 3 test tissue flasks, or from the control flask, respectively. Centrifugation at 5,000 r.p.m. for from 15 to 30 minutes was employed when fluid was titrated for virus content, or when neutralization tests were carried out. Simms' 3-X6 balanced salt solution¶ was the diluent utilized in titration and in neutralization tests.

Paralysis and death of the recipient mice were accepted as evidence for the presence of virus. When titrations were carried out to assess the extent of multiplication in successive tissue culture transfers, the Reed and Muench method was used to calculate the 50% end-points. The virus under propagation in each tissue culture series was tested at least once for its pathogenicity for monkeys.

TABLE II. Results of the Propagation of the Yale-SK Strain in Tissue Culture, Series B.

GROWTH OF POLIOMYELITIS VIRUS (YALE-SK)
IN CULTURES OF TESTICULAR TISSUE
FROM MONKEY

FLUID FROM	RESULTS IN MICE	LOG OF DILUTION OF ORIGINAL VIRUS BY FLUID REPLACEMENT									
		2.7 3.6 4.5	6.2 7.1 8.0	9.7 10.6 11.5	13.2 14.1 15.0	16.7 17.6 18.5	20.2 21.1 22.0	23.7 24.6 25.5	27.2 28.1 29.0	30.7 31.6 32.5	34.2 35.1 36.0
TISSUE FLASKS	PARALYSIS†	3/6*	4/8 4/6	1/6 2/6 5/7	1/7 2/8 4/7	0/5 3/6 3/6	0/6 3/7 3/6	3/6 4/6 3/6	0/6 3/6 5/6	0/4 3/6 1/6	2/5 2/6
	DEATH†	5/6	7/8 6/6	2/6 5/6 6/7	5/7 6/8 5/7	4/5 5/6 6/6	4/6 6/7 5/6	5/6 6/6 5/6	4/6 5/6 6/6	0/4 6/6 5/6	5/5 5/6
CONTROL FLASKS	PARALYSIS	0/6	0/4 0/6			0/6					
	DEATH	1/6	0/4 1/6			0/6					
		2.7	4.4	6.1	7.8	9.5	11.2	12.9	14.6	16.3	18.0
		LOG OF DILUTION OF ORIGINAL VIRUS BY TISSUE REPLACEMENT									

* Denominator signifies No. of mice inj. intracer., .03 ml. Numerator signifies No. that died.

† "Paralysis" signifies No. of mice paralyzed (one observation per 24 hr). Paralysis within 24 hr of inoculation was not included. "Death" signifies No. of mice dead, excluding those dying within 48 hr of inoculation.

Results. The 3 passage series, Series A (Lansing-monkey testicular tissues), Series B (Yale-SK monkey testicular tissue), and Series C (Yale-SK virus in human or monkey testicular tissue), had been carried through from 8 to 10 successive tissue changes and from 24 to 30 fluid changes when this report was written. The results of 2 of these 3 passage transfer series are made known in Tables I and II. The data, as presented, will be made easily understandable by a few comments. Evidence for the multiplication of virus necessarily must be determined by relating at any given passage transfer the dilution factor and the infectious titer, as measured by the LD_{50}. Accordingly, the presence of virus was measured at successive stages in each experimental series by the incidence of paralysis and death in the test mice. For interpretation of the results, the inoculum on transfer to each of the first set of flasks was diluted $10^{2.7}$, when one part by weight of virus-containing brain and cord was added to 529 parts of the mixture that made up the balance of flask content. Thereafter, the viral content was diluted at each subsequent tissue replacement by 1:53, or $10^{1.7}$. On the other hand, the calculation of the viral dilution that resulted from each replacement of supernatant fluid was not absolute, simply because of the mechanical difficulty of removing all fluid from beneath the cellophane membrane. However, when actual measurements were made of the residual fluid, the mean quantity was established as 0.7 ml. Accordingly, it was concluded that each fluid change by the addition of 5.0 ml of fresh nutrient fluid resulted in a dilution of 1:8, or $10^{0.9}$. However that may be, it can be seen from the data presented in Tables I and II that unequivocal evidence

TABLE III. Summary of Experimental Findings Showing Propagation of Poliomyelitis Virus in Tissue Culture.

Tissue culture series	Virus and cells employed	Tissue No.	Fluid No.	Dilution of original virus by: Tissue replacement	Dilution of original virus by: Fluid replacement	LD_{50}	Neutralization test
A	Lansing-monkey testicle	7	20	$10^{12.9}$	$10^{24.6}$	$10^{-1.5}$	
	Lansing-monkey testicle	7	21	$10^{12.9}$	$10^{25.5}$	$10^{-1.6}$	+
B	Yale-SK monkey testicle	7	20	$10^{12.9}$	$10^{24.6}$	$10^{-3.0}$	
	Yale-SK monkey testicle	7	21	$10^{12.9}$	$10^{25.5}$	$10^{-2.1}$	+
	Yale-SK monkey testicle	10	29	$10^{18.0}$	$10^{35.1}$	$10^{-1.8}$	
C	Yale-SK human-monkey testicle	8	23	$10^{14.6}$	$10^{28.1}$	$10^{2.4}$	

for the propagation of virus was established for each of the three experimental series, whether calculated by dilution from successive replacements of tissues, or from successive replacements of fluid. In contrast to this evidence for viral multiplication, the virus employed as inoculum quickly disappeared from the control flasks.

It can be seen from Table I that the calculated dilution of the original inoculum of the Lansing strain of virus was $10^{29.0}$ after 24 fluid replacements over a period of at least 120 days, or $10^{14.6}$ when calculated after 8 tissue replacements. Table II makes it known for Series B that the calculated dilution of the original inoculum of Yale-SK strain of virus, after 30 fluid changes, was $10^{36.0}$, or 10^{18} after 10 tissue changes. The third passage series employed the Yale-SK strain of virus, as in Series B, but differed by utilizing testicular tissues derived both from man and monkey. The results after 30 fluid changes and 10 tissue changes were in complete agreement with the findings which are presented in Table II for Series B.

The pathogenicity for monkeys of the products of tissue culture growth was tested for Series A and Series B after 6 tissue changes, and for Series C after 7 tissue changes, by the injection intracerebrally, 0.5 ml of supernatant fluid which had been pooled for 3 tissue flasks. The development in each of the recipient monkeys of fever, paralysis, and the histological alterations in the central nervous system of poliomyelitis was accepted as evidence that a virus of the mouse encephalomyelitis group had not been picked up inadvertently.

A summary of the findings at the 7th and 8th tissue replacement is presented in Table III.

Table III makes known the results (a) of LD_{50} determinations, and (b) of the neutralization tests which established the identity of the virus after 7 tissue passages in each culture series. The samples of specific antiserum were kindly supplied by Dr. Jonas Salk. Specific identification of the Lansing virus was established by monkey serum labeled "Lans. Antisera Pool A-300. 20 June 1949"; the Yale-SK virus was identified by monkey antiserum labeled "YSK Aliquot Pool B-532—6/15/50."

Summary. In experiments in which monkey or human testicular cells in tissue culture were infected with poliomyelitis virus, Lansing and Yale-SK strains, proof was obtained that the virus in 3 passage series had been propagated. At the time this report was written it had been established for these 3 passage series that the minimal dilution factor based on tissue replacements ranged from $10^{14.6}$ to 10^{18} and, when assessed by fluid replacement, from 10^{29} to 10^{36}. The LD_{50} of each strain of virus was determined on successive transfers, and the identity of each strain of virus was established by neutralization tests and histopathological findings in monkeys dead from the injection of tissue culture virus. Control experiments and other tests made known

that propagation of poliomyelitis virus did not occur in the absence of viable testicular cells and that an extraneous virus was not inadvertently acquired during the course of these studies. It is plain that the results of the present investigation substantiate the observations of other workers that poliomyelitis virus can propagate in extraneural tissues. From these findings it is apparent that the assumption of an obligate neurotrophism for poliomyelitis virus no longer is tenable. This observation and the demonstration regularly of the natural occurrence in poliomyelitis of virus in the oro-intestinal tract emphasize the need for a renewal of investigative studies of the factors that make possible invasion of the central nervous system to result in the overt and residual manifestations of poliomyelitis.

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Further Studies on Experimental Parasystole and Extrasystoles in Groups.*

(18666)

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In parasystole a center in one chamber of the heart forms rhythmic stimuli, undisturbed by the basic cardiac rhythm or by any other stimulus spreading over the heart. This leads to a regular interference of the basic and the ectopic rhythm. An experimental study of this interesting arrhythmia, which is not too rare in man, was impossible because no method was available to provoke it regularly.

Recently, we reported that the subepicardial application of veratrine alkaloids regularly leads to the appearance of parasystole, lasting for a few minutes, and showing all its characteristic features(1). In this paper another method will be reported for eliciting parasystole with ease. It consists of applying mechanical or electrical stimuli to the ventricles of the dog's heart after the intravenous administration of acetylcholine and complete atropinization.

Method. All 18 experiments were performed on dogs with the same technic as reported in previous investigations of the senior author in this journal. The electrocardiograms were taken in lead 2. A fresh solution of acetylcholine chloride "Roche" was used in all experiments. The amount of 0.05 g of acetylcholine was injected intravenously in 3-4 divided doses within 10 minutes and 0.2 mg per kg of atropine sulfate was given intravenously shortly afterwards. When acetylcholine was administered without atropinization, mechanical or electrical stimulation of the ventricles initiated ventricular fibrillation(2).

Results. Following the injection of acetylcholine and atropine, the mechanical stimulation of the ventricles by a stroke with a blunt instrument or the application of a few induction shocks provoke a heteropic ventricular tachycardia originating in the stimulated area. In 15 out of 18 experiments a parasystole was observed; in 3 experiments a regular ectopic tachycardia appeared, which persisted for 45 to 160 seconds and then reverted to regular sinus rhythm.

Fig. 1 shows a representative electrocardiogram exhibiting parasystole. A short part of the tracing between Fig. 1a and Fig. 1b is omitted. Fig. 1b and Fig. 1c are continuous. The dog had received acetylcholine and atropine in the doses mentioned above. The stormy changes caused by the acetylcholine, with the primary inhibition of the heart and the secondary increase of rate and motility had subsided. At the beginning of Fig. 1a a regular sinus tachycardia exists with a rate of

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* This investigation was supported (in part) by a research grant from the National Heart Institute, U.S. Public Health Service.

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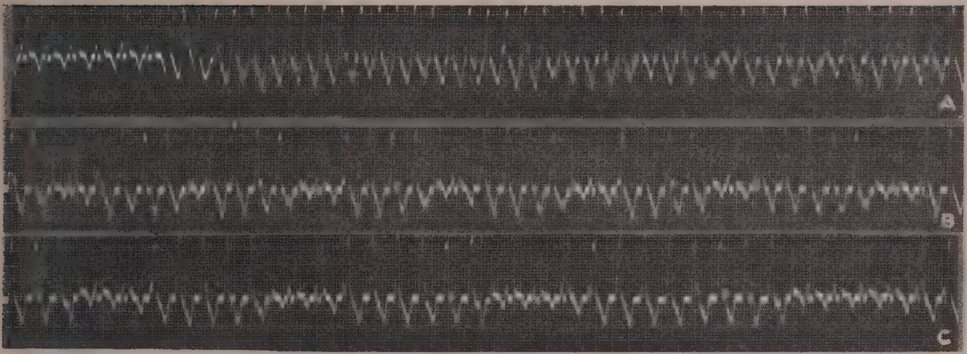

FIG. 1.
Parasystole following administration of acetylcholine and atropine with stimulation of the ventricle.

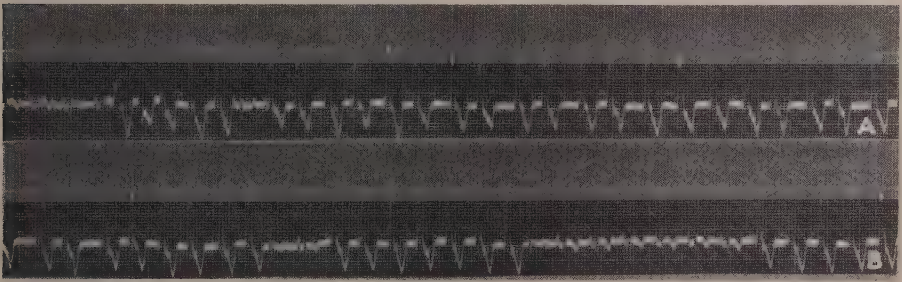

FIG. 2.
Parasystole with group formation of the ectopic beats.

188 beats per minute. Mechanical stimulation of the right ventricle elicited 2 ventricular extrasystoles followed by a chain of spontaneous ectopic beats with a rate of 200. This tachycardia gradually slowed down and occasionally (the second, the seventh, the twelfth, and the sixteenth beat counted from the end of the tracing in Fig. 1a) a sinus beat succeeds in activating a greater or lesser part of the ventricles, so that combination beats appear. When the ectopic stimulus formation slowed down, more sinus beats reached the ventricle and a typical interference of both rhythms appeared. In Fig. 1b, one or 2 sinus beats appeared in succession between groups of 4 ectopic beats. The length of one period of the ectopic rhythm measured 0.28 second. The duration of the pauses between ectopic beats, filled with sinus beats, is 0.56 second when only one sinus beat appears, and 0.85 second when 2 sinus beats follow each other. In Fig. 1c the length of an ectopic period in the beginning of the tracing measured 0.28 second and at the end it is 0.32 second. In 4 instances 3 to 4 sinus beats or combination beats are seen between the groups of ectopic beats. The length of these pauses between the groups of ectopic beats measure: 1.13 (28 x 4), 1.19, 1.19 (29.7 x 4), 1.84 (30.6 x 6). Accordingly, we are dealing with a center which is protected, the rhythmic stimulus formation proceeding without disturbance by the sinus beats.

The appearance of combination beats and the longer pauses between ectopic beats having the length of a multiple of a single ectopic period, were noted in all experiments in which parasystole could be elicited.

Fig. 2 shows a tracing obtained after stimulation of the right ventricle by a few induction shocks. Previously acetylcholine and atropine had been given in the same amount as in the experiment discussed earlier. Three extrasystoles resulting from the inductian shocks (Fig.

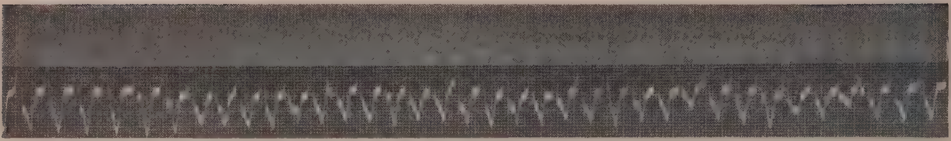

FIG. 3.
Increase of rate of the ectopic tachycardia on warming the focus of origin.

2a), are followed by 2 spontaneous ectopic beats which succeed each other at a distance of 0.32 second. Then a sinus beat caused a period lasting 0.64 second free of ectopic beats. Parasystole is present, because the sinus beat does not disturb the rhythm of the ectopic center. A few seconds later successive ectopic beats show distinct group formation, a short interval of 0.33 second being followed by one of 0.44 second. In Fig. 2b (Fig. 2a and Fig. 2b are continuous) the successive ectopic beats again appear regularly (period: 0.38-0.34 second). At one place the chain of ectopic beats is interrupted by 2 successive sinus beats. The period free of ectopic beats measures 1.06 (0.35 x 3) second and later another pause filled by 7 sinus beats measures 3.12 (0.346 x 9) second.

To prove that these ectopic beats actually originated in the area to which stimuli had been applied and also prove that they are due to stimulus formation in a center and not to a re-entry mechanism, we warmed the point which had been stimulated. This invariably increased the rate.

Fig. 3 shows an ectopic rhythm obtained after the intravenous injection of acetylcholine and electrical stimulation of the conus of the right ventricle with a few rapid stimuli from an electrodyne stimulator. Ectopic beats appeared with a rate of 150-166 (periods of 0.40, 0.36, 0.36, 0.37 second). Warming the stimulated area increased the rate to 200. At the end of Fig. 3, on 2 occasions, sinus beats reach the ventricle without disturbance of the ectopic center. The two pauses free of ectopic beats measure 0.62 second; consequently they represent a simple multiple of an ectopic period.

Discussion. Abnormal ectopic stimulus formation occurs under the influence of acetylcholine. It is more pronounced in the auricles, where fibrillation is a common event(2).

The experiments just discussed show that in the atropinized heart under the influence of acetylcholine, typical parasystole is induced by electrical or mechanical stimulation, with all characteristic signs, namely, changes of the length of coupling, protection of the ectopic center and combination beats. Of importance is the fact that in these experiments extrasystoles with fixed coupling were not observed. Fixed coupling appeared readily after topical application of barium, sodium chloride, digitalis, or strophanthin as previous experiments have shown(3,4). One may conclude therefore that in parasystole a special type of stimulus formation occurs and that the heart exhibiting a parasystolic stimulus formation need not show an extrasystolic one.

Of interest is the appearance of ectopic beats in groups, with alternation in length of diastole, during parasystole (Fig. 2), as described before by one of us(5). This demonstrates conclusively that stimulus formation in the heart, like the spontaneous firing of impulses in nerve and muscle(6,7) may occur in groups of "multiple responses" and still originate in a focus.

Conclusions. Short mechanical or electrical stimulation of the dog's ventricle, following the administration of acetylcholine and atropine, leads to the temporary appearance of parasystolic arrhythmias. The ectopic parasystolic center may form stimuli in groups.

Our thanks are due to Mr. Max Weinstein whose generous gift enabled us to purchase the Electrodyne Stimulator used in this investigation.

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Further Observations on the Anesthetization of Rabbit's Cornea by Non-Surface Anesthetics. (18667)

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Recently Greig and coworkers(1,2) have reported that the simultaneous or prior administration of topical physostigmine allowed procaine to become an effective topical anesthetic and they hypothecated that this effect was due to the inhibition of cholinesterase by the physostigmine. The theoretical and practical importance of this effect, coupled with the thought that the altered procaine response might be due to a local action of the physostigmine independent of its actions on cholinesterase prompted the present studies upon this phenomenon. On the basis of the Greig hypothesis, the altered procaine response should be independent of the route of administration of the physostigmine. The results obtained in an initial study in which physostigmine was given subcutaneously indicated the advisability of an attempt to repeat and extend the observations of Greig *et al.*(1).

Experimental. The rabbit's cornea *in vivo* was utilized for testing surface anesthesia, and the absence of corneal reflex after touching the cornea with a stiff hair was utilized as a criterion of anesthesia. The drugs (0.5 ml in the concentration and solvent listed in Table I) were instilled into the rabbit's eye and the eyelids held shut for 30 seconds. Subsequently the presence or absence of a corneal reflex was tested at intervals. In the course of these studies a total of 1320 observations were made on the eyes of 44 rabbits. In all cases the observers were unaware of the particular agents given to any one rabbit. In 504 of these observations on 18 rabbits by 11 individuals the observers were completely unaware of the nature of the experiments. The primary data are listed in Table I wherein a plus (+) indicates the presence of corneal anesthesia. The data were statistically analyzed utilizing the method of χ^2 to test the null hypothesis(3).

Results and discussion. The first experiments revealed that subcutaneous physostigmine alone (No. 5) caused significant corneal anesthesia when compared with the control (Nos. 1, 2, 3) ($p < 0.001$) and, further, that the presence or absence of procaine in conjunction with this treatment did not significantly alter the degree of corneal anesthesia (Table I—compare No. 5 vs. No. 6; $p > 0.99$). The presence of significant corneal anesthesia, as tested by this method, in the absence of a local anesthetic suggested further experiments in order to clarify this effect of physostigmine. Accordingly we have tested pilocarpine, a "true" parasympathomimetic agent, and strychnine, a cord stimulant. As may be noted in Table I, strychnine did not alter the action of procaine. Subcutaneous pilocarpine by itself (No. 7) exhibited a significant degree of corneal anesthesia when compared with the control situations (Nos. 1, 2, 3) ($p = 0.035$). As with physostigmine, procaine did not significantly alter the response (No. 7 versus No. 8) ($p = 0.31$).

In view of the above findings it was felt that it would be of value to repeat portions of the work reported by Greig *et al.*(1). In the first of these experiments 0.9% saline solution was utilized as the drug solvent. However, noting the deviations of the present results from those of the previous workers, the experiments were again repeated using water as the drug solvent. Pooling the results, and comparing water as a solvent in all situations (Table I—Nos. 2, 12, 13, 15) against 0.9% saline as a solvent in all situations (Table I—Nos. 3, 11, 14, 16) reveals that there is no significant difference between the solvents as far as the results of this experiment are concerned ($p = 0.99$). In view of this finding,

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TABLE I. Incidence of Corneal Anesthesia.

No.	Treatment	+*	—	% showing corneal anesthesia
1	Control (no drug)	1	41	2
2	Water	11	77	13
3	Saline 0.9%	18	92	16
4	Pontocaine 0.5% ophthalmic	37	29	56
5	Subcut. physostigmine 1 mg/kg	61	56	52
6	” ” ” + procaine (2% in saline)	63	54	54
7	Subcut. pilocarpine 5 mg/kg	13	35	27
8	” ” ” + procaine (2% in saline)	20	28	42
9	Subcut. strychnine .25 mg/kg	3	45	6
10	” ” ” + procaine (2% in saline)	4	44	8
11	Procaine (2% in saline)	8	100	7
12	” (2% in water)	8	58	12
13	Physostigmine (.1% in water)	2	64	3
14	” (.1% in saline)	3	90	3
15	” (.1% in water) + procaine (2% in water)	8	58	12
16	Physostigmine (.1% in saline) + procaine (2% in saline)	15	78	16
17	Zephiran 1:3500	3	45	6
18	” 1:3500 + procaine (2% in saline)	9	39	19
	Totals	287	1033	

* (+) indicates absence of corneal reflex.

the data have been regrouped by combining the results with the separate solvents in order to test the effect of the combination of topical physostigmine and procaine against the effects of topical procaine alone (Table I—Nos. 11, 12 vs. Nos. 15, 16) ($p = 0.34$). The result of this analysis reveals that the combination of topical physostigmine with procaine, in these experiments, does not produce a significant difference in corneal anesthesia. Further, Zephiran, an agent used quite commonly to increase the penetration of agents into the eye, does not alter the action of procaine (Table I—No. 17 vs. No. 18; $p = 0.165$). As a control measure, pontocaine was shown to exhibit surface anesthesia (Table I—No. 4 vs. all other situations; $p < 0.001$).

In a further study of the possible mechanism of the production of corneal anesthesia following subcutaneous administration of physostigmine and pilocarpine, the following supplemental observations were made on rats. Carbaminoylcholine produced a similar response and the effects of all three were blocked by atropine given either before the drug or after the corneal anesthesia had been produced. Epinephrine given subcutaneously did not produce corneal anesthesia, and adrenalectomy did not alter the response to physostigmine or pilocarpine. It appears that the corneal anesthesia noted following these autonomic drugs is related to their muscarinic effects.

The results of the present experiments, in which no demonstration of an action of physostigmine, either topically or systemically (Table I—compare No. 5 vs No. 6 and Nos. 15 and 16 vs Nos. 11 and 12), on the topical efficacy of procaine was noted, lead one to question the hypothesis put forward by Greig and her coworkers relating cholinesterase inhibition to the penetration of topical anesthetics. Further, upon examination of the data relating surface anesthesia to the cholinesterase inhibiting powers of the various local anesthetics on dog erythrocytes (Greig *et al.*) (1), it is apparent that the concentrations used *in vivo* are for procaine 53 times, orthoxine 70 times and mesidicaine 25 times the highest concentrations needed in the *in vitro* experiments to produce significant cholinesterase inhibition. In view of the fact that for most cholinesterase inhibitors the *in vitro* concentrations are much higher than those required *in vivo,* it would appear that even in the absence of physostigmine the local anesthetics should produce significant cholinesterase inhibition in the concentrations used *in vivo*. Following Greig's hypothesis, therefore, these agents, in the concentrations used should produce significant topical anesthesia. However, the validity of the comparison of quantitative data relating the cholinesterase of dog erythrocytes with that of rabbit cornea may be questionable. Nevertheless, in the light of the experimental data presented in this study and the above considerations, it is felt the probability of cholinesterase playing a role in the penetration of topical anesthetic agents is quite small.

Summary. In the present experiments, utilizing 1320 observations on the eyes of 44 rabbits, physostigmine, either topically or subcutaneously, did not alter the topical anesthetic activity of procaine. Subcutaneous administration of physostigmine or pilocarpine produced corneal anesthesia in the rabbit eye as measured by loss of corneal reflex. The role of cholinesterase inhibition in the penetration of non-surface anesthetics is discussed.

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Mammary Spreading Factor and Relaxin.* (18668)

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In a previous paper(1) data were presented indicating the presence of a factor (enzyme?) in the growing mammary gland of the rat which was believed to facilitate the forward advance of the duct and alveolar structures into the subcutaneous tissue in which the glands are located. This factor, which had spreading properties, was not identical with hyaluronidase.

Hamolsky and Sparrow(2) reported that relaxin synergized the action of estradiol and progesterone in stimulating mammary gland growth and Van der Meer(3) suggested that relaxin might act as a spreading factor. It seemed of interest to determine whether there might be a relation between the spreading factor present in the growing mammary gland during pregnancy and relaxin. If so it would aid in explaining the possible role of relaxin in mammary gland growth and further define the properties of the mammary gland spreading factor.

Methods and Materials. The relaxin used in these experiments was prepared as an aqueous extract of swine ovaries containing 75 guinea pig units per ml.† Extracts(1) of mammary glands from albino rats pregnant 8 to 14 days were employed to determine the ability of the mammary spreading factor to cause pelvic relaxation. Immature female guinea pigs (pretreated with estrogen) were employed as assay animals. Pelvic relaxation, used as the index of relaxin activity, was determined by palpating and floroscopically examining the guinea pigs preceding and 7 or 8 hours following injection of the test material.

Experimental and results. In testing the spreading properties of the relaxin sample eight portions of 0.2 ml were injected together with 0.1 ml of dye. The average area of spread was 231.0 sq mm. Testicular extracts and pH 6.0 buffer solutions used as controls

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* Contribution from the Department of Dairy Husbandry, Mo. Agr. Exp. Station, Journal Series No. 1253. Approved by the Director of the Mo. Agri. Exp. Station.

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† The relaxin used in this investigation was generously provided by Dr. R. L. Kroc, Chilcott Laboratories, Morris Plains, N. J.

TABLE I. Spreading Tests.*

Assay material	Initial bleb, mm	After 30 min., mm	Area of spread, sq mm
pH 6 buffer	14 × 14	16 × 17	59.7
	14 × 14	16 × 16	47.1
	14 × 14	16 × 16	47.1
	14 × 14	16 × 17	59.7
			Avg 53.4
Testicular extr.	13 × 14	24 × 24	309.6
	14 × 14	24 × 24	298.6
	14 × 14	25 × 25	317.4
	14 × 14	23 × 25	298.6
	14 × 14	24 × 24	298.6
			Avg 304.6
Relaxin†	14 × 14	21 × 22	210.6
	13 × 14	21 × 23	237.3
	14 × 14	21 × 22	210.6
	14 × 14	22 × 22	226.3
	13 × 14	21 × 22	221.6
	14 × 14	21.5 × 22	235.7
	14 × 14	22 × 23	245.1
	14 × 14	23 × 23	260.8
			Avg 231.0
Relaxin after 10 min. at 40°C‡	14 × 14	22 × 23	245.1
45°C	13.5 × 14	22 × 22	232.6
50°C	14 × 14	17 × 18	90.0
55°C	14 × 14	16 × 18	72.8

* Injections included 0.2 ml assay material + 0.1 cc dye.

† Releasin 31-81DSF-75GPU/ml.

‡ 5 samples used at each temperature.

gave areas of spread of 304.6 and 53.4 sq mm., respectively.

Tests were then made to determine if the spreading factor was heat labile. Ten portions of the relaxin sample, in two equal groups, heated to temperatures of 40°C and 45°C for 10 minutes showed the spreading factor still active while 2 other groups of 5 portions each showed no spreading activity after being heated to 50°C and 55°C for the same length of time (Table I).

Determination of the hyaluronidase content of relaxin was accomplished by assaying three portions turbidimetrically. Results of the assays indicated that in no case was the substrate, hyaluronidate, hydrolyzed by relaxin.

As the relaxin preparation indicated the presence of a heat labile spreading factor a portion of the sample was heated to 60°C for 10 minutes. After spreading type tests had shown the spreading factor to be inactivated, the ability of the heated portion to cause pelvic relaxation was determined. Ten pretreated guinea pigs were injected with 5.0 G.P.U. of the heated relaxin. Ten other pretreated guinea pigs were injected with 5.0 G.P.U. of the unheated sample. Apparently all or nearly all of the original activity was retained for the pelvic relaxation was as great in guinea pigs injected with heated portions of the relaxin preparation as in guinea pigs injected with the unheated solution.

Finally the mammary spreading factor was tested for its ability to cause pelvic relaxation. In all, 21 samples containing mammary spreading factor were tested. The first trial involved injection of 5 samples of 0.3 ml into 5 pretreated guinea pigs. In the second series of 5 samples the amount injected was raised to 0.5 ml while the amount injected in the last 11 trials was 1.0 ml. Examination of the injected guinea pigs showed little or no relaxation in response to all levels of the mammary spreading factor which were tested.

Discussion. While the experimental results show rather conclusively that the mammary spreading factor did not cause pelvic relaxation in the guinea pigs, the results obtained with the relaxin preparation are not so easily evaluated. These data indicate the presence of a heat labile spreading factor in the relaxin preparation.

Heat inactivation (60°C for 10 minutes) of the spreading factor failed to diminish the ability of the relaxin preparation to cause pelvic relaxation. Kroc(4) employed even higher temperatures in investigating the heat resistance of relaxin. He found that extracts similar to those employed in these experiments retained all or nearly all their "relaxin" ability after being heated to 90°C-95°C for 10 minutes or autoclaving at 15 lb pressure for 15 minutes.

These data indicate that the mammary spreading factor and relaxin differ both in heat tolerance and biological activity. Further it appears that the spreading factor present in the relaxin preparation plays no part in the relaxation phenomena and that the hormone possessed little or no ability to increase dermal permeability. Therefore, it is probable that the ovarian extract spreading factor was merely present in the relaxin extract and had no connection with the hormone.

It seems possible that the relaxin extract employed by Hamolsky and Sparrow might contain a spreading factor similar to the one observed in this study. There are also possibilities that ovarian extracts of relaxin now in use might contain other biologically active components. Therefore, it is suggested that in future experiments care should be taken to determine whether the synergism of relaxin preparations with estrogen and progestin which produced increased growth of the mammary is due to relaxin, the spreading factor or an undetermined substance.

Summary. 1. It was observed that a relaxin preparation extracted from swine ovaries contained a spreading factor which was inactivated by heating at 50°C for ten minutes. The inactivation of the spreading factor did not diminish the effectiveness of the relaxin preparation to cause pelvic relaxation. 2. The spreading factor extracted from the mammary glands of pregnant rats had no ability to cause pelvic relaxation in the amounts employed.

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Effect of Administration of Intermedin upon Melanin Content of the Skin of *Rana pipiens*. (18669)

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The relation of humoral factors, especially of the pars intermedia, in controlling the color responses of amphibia have been clearly elucidated by the investigations of Hogben, *et al.*, (1,2). Although the melanophore hormone* is also considered to be involved in melanin synthesis(4), evidence for its role in influencing the actual production of melanin is less direct. Odiorne(5) observed significant decreases in total numbers of melanophores and melanin granules in fundulus maintained for long periods on light backgrounds, as well as a reversal of this effect when the animals were placed on a black background. Sumner (6,7) measured changes in melanin content of the skins of several species of fish as a function of background albedo, noting that both melanin content and total melanophore number decreased on exposure to light backgrounds. Dawes(8) obtained similar results using *Rana temporaria;* in addition, he showed that prolonged dark adaptation resulted in an actual increase in melanin content. Direct evidence for the implication of intermedin as a factor in melanin formation is limited to a single experiment performed by Dawes. Perfusion of the severed left hind legs of 6 frogs with posterior pituitary extract resulted during 48 hours in a 33% increase in melanin content of these, as compared to control limbs.

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* Although some doubt exists as to the identity of intermedin, the name given by Zondek(3) to the erythrophore-expanding substance of the pituitary, with the melanophore hormone, the two terms are used interchangeably.

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The rapidity of the effect observed in these perfusion experiments, especially when contrasted with the slow increases observed with intact animals, suggested the desirability of a reinvestigation of the effect of exogenous intermedin upon melanin synthesis in frogs. It has been found that prolonged administration of intermedin preparations to frogs maintained in surroundings of neutral albedo results in an initial *decrease* in melanin content. This is followed by a slow increase which results, after a total of 8 weeks, in melanin levels approximately 40% above those of control animals.

Experimental. Intermedin preparations. Whole pituitaries of hogs, stored frozen until used, were extracted with dilute (0.25%) acetic acid at 90°C. After removal of the insoluble residue, ethanol was added to the filtrate to a final concentration of 70%. The inactive precipitate was removed, and the ethanol content of the filtrate was raised to 90%, whereupon most of the activity precipitated. The precipitate was redissolved in water and the solution stored in the frozen state. Melanin determinations were made essentially according to the method of Dawes. As a check upon the reproducibility of the method, each skin, after removal from the animal, was divided into 2 approximately equal parts. Each half was weighed, boiled in distilled water for 15 minutes, and finally digested in 2% pepsin at pH 1.2 for 48 hours at 48°C. The undissolved residue was washed twice with water and then extracted with two 50 ml portions of hot 1M NaOH. After diluting the combined alkaline extracts to 100 ml, the transmission of the resulting solution was read in a Klett colorimeter, equipped with a filter having maximum transmission at 540 mμ. The results have been expressed, arbitrarily, by dividing the observed extinction by the weight of skin used. In determinations of the melanin content of the skins of twenty-seven control animals, the average discrepancy between the two values for each animal was 2.1%; the greatest difference encountered was 5.0%. Seventy-two frogs (*Rana pipiens*) were divided into 2 groups containing, respectively, 24 control and 48 experimental animals; the average body weight was the same for each group. They were kept in gray stone aquaria under ordinary laboratory lighting conditions (out of direct sunlight) at 15-20°C. All but 3 survived for the duration of the experiment (8 weeks).

Appropriate preliminary experiments indicated that a single injection of 1500 units† of intermedin was required to effect maximal darkening which would persist for 24 hours. To ensure a sufficiently high level of the hormone, about 9600 units, contained in 0.1 ml of solution, was injected into each experimental animal daily. The controls were injected with an equal volume of normal saline. Groups of control and experimental animals were killed at intervals of two weeks for determination of total melanin.

Results. It was found (Table I) that the injection of relatively large quantities of intermedin results in a decrease in melanin content during the first four weeks of the experiment. The difference between control and experimental values at 2 weeks is of doubtful significance ($p = 0.13$); at 4 weeks, however, there is a real difference ($p < 0.01$) between the means of the 2 sets of determinations. This trend is rather quickly reversed, so that at 6 weeks the 2 groups are practically

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8. Dawes, B., *J. Exp. Biol.*, 1941, v18, 26.

† The unit here referred to is defined by Frieden, Fishbein and Hisaw(9), and is approximately equal to the intermedin content of 0.1 microgram of standard posterior lobe powder.

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TABLE I.

Period of treatment (weeks)	No. of animals		Avg body wt		Melanin contents*			
	control	treated	control, g	treated, g	Control	Treated	Difference	p
2	5	10	36.1	38.0	.176 ± .051	.147 ± .020	—.029	.13
4	6	12	36.2	39.7	.154 ± .020	.125 ± .011	—.029	$<.01$
6	5	13	40.3	36.9	.140 ± .020	.147 ± .019	+.007	$>.50$
8	7	11	40.5	36.4	.138 ± .017	.200 ± .023	+.062	$<.01$

* Melanin contents are expressed in terms of relative color intensity per g of skin. Each value is accompanied by an estimate of its standard error.

identical with respect to melanin content. The value for the treated group then raises rapidly, reaching 41% above the control level at 8 weeks, at which time the experiment was terminated.

The downward trend observed in the pigment content of the skins of control animals during the 8 weeks of the experiment is accompanied by a narrowing of the range of individual values. This may be attributed to the fact that of the heterogeneous group of animals initially selected some were intensely pigmented; these would be likely to lose pigment during the time they were exposed to the experimental environment, which included a gray background and relatively low average illumination intensity. Initially paler animals, on the other hand, would be expected to undergo little change.

Assays of the pituitary glands of control and experimental animals after the eighth week indicated that the hypophyses of animals receiving exogenous intermedin responded by a considerable decrease in total intermedin. Thus, the pituitaries of untreated animals averaged 525 units per gland, whereas the corresponding value after eight weeks of treatment was 140 units per gland.

Discussion. Our results are in decided contrast to the rapid changes obtained in Dawes' perfusion experiments, but are in general qualitative agreement with his background studies.

Perhaps the most surprising result of this investigation was the initial relative decrease in melanin observed. It is conceivable that dispersion of pigment within the melanophores facilitates the normal metabolic processes by which the pigment is degraded; if so, this must occur before the melanin-synthesizing mechanism is significantly accelerated.

It is not yet clear whether the increase in melanin which follows prolonged administration of intermedin is referable directly to the increased concentration of hormone in the tissues, or is an indirect effect resulting from long-continued dispersion of pigment granules within the chromatophores. It is quite likely that oxidation of melanin precursors may be facilitated by an increase of effective surface: volume ratio of pigment. It might be possible to settle this question by varying the amount of hormone injected.

Summary. The administration of intermedin, prepared from hog pituitary glands, results in a decrease, after four weeks, of twenty per cent in the melanin content of the skin of *Rana pipiens*. Subsequently, there is a rapid increase, reaching levels 40% above normal after a total of 8 weeks.

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Morphology of Emetic Chemoreceptor Trigger Zone in Cat Medulla Oblongata. (18670)

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Recent experimental analysis of the nervous regulation of vomiting by Wang and Borison (1) represents a major revision of the traditional concept of the emetic mechanism. Contrary to the prevailing belief that certain emetic agents such as apomorphine and digitalis glycosides induce vomiting by direct excitation of the vomiting center, these workers have demonstrated that the drugs mentioned act on a separate locus situated in the floor of the IVth ventricle and designated by them as the "chemoreceptor trigger zone for emesis"(1,2). They localized this trigger zone in the vicinity of the ala cinerea by studying the effects of chronic lesions placed in the medulla oblongata of dogs with the aid of electrocautery. By trial and error the size of the lesion was reduced to the smallest permissible limits consistent with the elimination of the vomiting induced by centrally-acting emetic drugs. In this manner successful preparations were obtained in which most of the dorsal vagal nuclei remained intact. It was concluded by Wang and Borison that the trigger zone is situated in the dorsolateral portion of the ala cinerea since this portion was consistently destroyed by their operations.

Because of its widespread visceral connections, the ala cinerea has long been suspected of having a role in the regulation of vomiting, and for many years it was actually considered to be the site of the vomiting center(3). This contention proved to be wrong when the vomiting center was shown to be situated in the lateral reticular formation of the medulla (1). Nevertheless, the ala cinerea retained some measure of its original importance with its newly acquired distinction as the probable location of the emetic trigger zone.

Histological examination of the periventricular medullary substance was made by us to characterize that portion of the ala cinerea which purportedly had the unique function of a chemoreceptor emetic trigger zone. No morphologically distinguishable structure could be identified in the dorsolateral portion of the ala cinerea. In the cat, there exists a highly vascular ependymal layer(4) which immediately overlies the dorsal vagal nuclei. This vascular ependymal layer has been signified by many workers(5-8) as the area postrema. In the present report, the term "ala cinerea" will be used only in reference to the grey matter embodying the dorsal vagal nuclei. Close study of the area postrema has revealed along its lateral margin a triangular zone which dips inwards between the ala cinerea and the descending vestibular root. This triangular zone is an essentially non-neural structure which, to the authors' knowledge, has not been previously described. This structure, which is now considered by us to be the chemoreceptor trigger zone, will hereinafter be designated as the CT zone. To examine the hypothesis that the CT zone may be the actual site of attack by central emetic drugs, the experimental study reported below was undertaken.

Experimental. The CT zone comprises less than a cubic millimeter of tissue on each side of the IVth ventricle. Because of the obvious difficulty in making accurate bilateral lesions restricted to such a minute area, localization of the emetic chemoreceptor function was attempted by seeking the common

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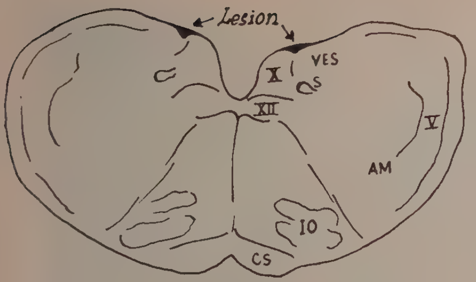

FIG. 1.

Cross-section of cat medulla oblongata above obex. Drawing made from projection of section from operated specimen. Animal died but did not vomit following intravenous ouabain 0.06 mg/kg, 10 days postoperatively. VES, descending vestibular root; S, fasciculus solitarius; AM, nucleus ambiguus; IO, inferior olive; CS, corticospinal tract.

topographical correlate in a series of cat preparations. With the use of electrocautery, lesions were placed in the region of the CT zone in 10 cats, all of which failed to vomit postoperatively to normally effective emetic doses of one or more of the cardiac glycosides. Prior to testing the operated animals, it was established that 0.08 mg/kg lanatoside C, 0.08 mg/kg scillaren A or 0.06 mg/kg ouabain will almost invariably induce emesis in the normal cat within 30 minutes after intravenous injection. Of 15 trials with the above mentioned drugs in the 10 operated cats tested as long as 3 months postoperatively at dose levels at least as high as those indicated, there was only a single vomiting response in one animal; this emesis occurred after an atypical latent period of 8 hours. Four of these cats died within half an hour following injection of the cardiac glycosides, yet no vomiting was elicited. Apomorphine was not used in the present experimental study since in the cat it is more prone to induce fits of hyperexcitability and combativeness than to elicit emesis. Histological study of the brains of 6 of the operated animals, including 3 which died as a direct result of the cardiac glycoside administration, showed that, while there was a variable degree of damage in the ala cinerea, the area postrema and the vestibular root, these structures were largely spared (Fig. 1). The CT zone, however, was found to be destroyed in all 6 brains examined.

Anatomical. In the cat the CT zone is seen in cross section as a more or less triangular structure, the apex of which extends for a short distance into the substance of the medulla between the ala cinerea and the descending vestibular root (Fig. 2). It is contiguous medially with the tissue which forms the main body of the area postrema. The CT zone is approximately one mm in length rostrocaudally. Rostrad, it extends a short distance beyond the rostral limits of the bulk of the area postrema; at this level the triangular configuration is particularly evident since the structure lies in direct relation medially to the dorsal sensory nucleus of the vagus and laterally to the vestibular complex. The caudal end of the CT zone has not been definitely determined but it appears to begin shortly rostral to the caudal edge of the area postrema which becomes evident at the obex.

In all preparations numerous small blood vessels enter the medulla between the CT zone and the vestibular complex or pierce the surface of the zone directly. These vessels give rise to small branches which form an extensive network in the area. The ependymal epithelium exhibits the same morphological modifications in the region of the CT zone as it does over the area postrema(7).

A variety of staining technics applied to the CT zone have revealed that it is essentially a non-neural zone occupied largely by fibroblasts and cells resembling protoplasmic astrocytes and astroblasts. Preparations appropriately stained for microglia, oligodendroglia and fibrous astrocytes revealed few such elements in this area. The vascular feet of the "protoplasmic astrocytes" are especially abundant in the CT zone. These appendages vary in form from thick single podia to rather long complexly branched processes. In addition to the "protoplasmic astrocytes" and the fibroblasts, a few small clusters of irregularly shaped cells with nuclei of similar size and form as those of ependymal cells were found scattered throughout the CT zone. The cytoplasmic bodies of these cells stained deep red in trichrome preparations. Collagen fibers are abundant and are found in close association with the numerous small blood vessels in the area.

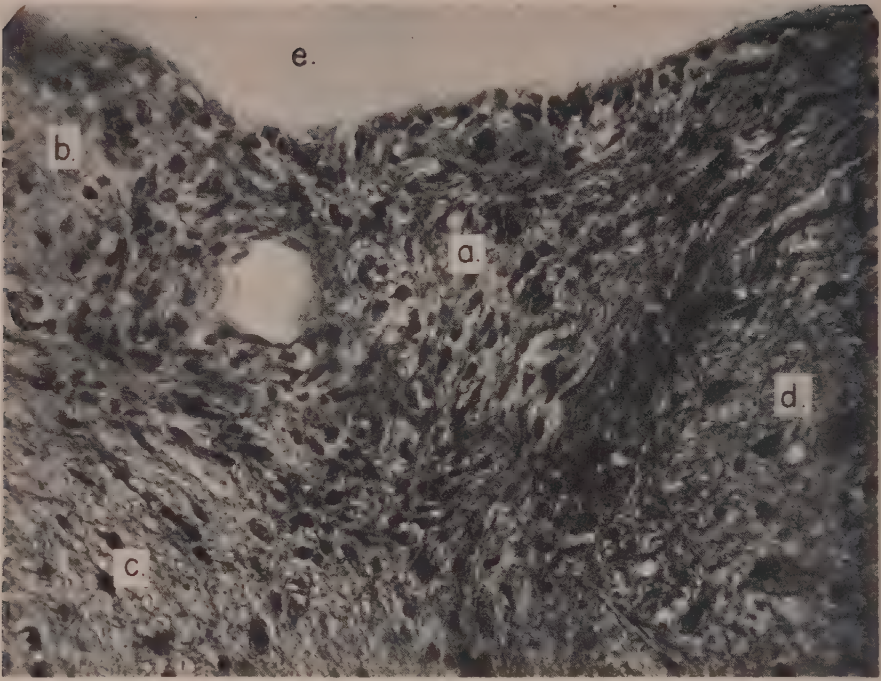

FIG. 2.

Photomicrograph of region of the CT zone. a. CT zone, the triangular area bounded by b, c, and d; b. area postrema proper; c. dorsal sensory vagal nucleus; d. descending vestibular root; e. fourth ventricle. Masson trichrome stain. Magnification approximately 500 diameters.

One silver preparation revealed the presence of fine nerve fibers in the CT zone. Many of these fibers course irregularly through the zone or between this structure and the adjacent vagal and vestibular nuclei. More discrete groups of fibers pass to the apex of the zone and emerge as loose bundles extending deeply towards the nucleus of the fasciculus solitarius. These bundles are joined by similar but smaller groups of fibers from the main body of the area postrema. Many fibers of the CT zone were observed to terminate in fine nerve endings close to the nuclei of the "protoplasmic astrocytes." Others appeared to end "freely" or adjacent to smaller nuclei, the identity of which has not been established.

The area postrema closely resembles the CT zone in its cellular composition but differs in that it generally stains less densely in hematoxylin and eosin and in Masson trichrome preparations and in that it contains scattered small neurons. The present findings, as concern the non-neural elements of the CT zone, are in rather close agreement with those of King's study of the area postrema in the cat(7). However, we have observed a greater abundance of vascular feet in the CT zone than he has described for the closely associated area postrema. We also take exception to King's conclusions in that we found numerous nerve fibers in the area postrema as well as in the CT zone. The directional organization of these fibers was more evident in the CT zone. For a survey of the literature regarding the area postrema, the reader is referred to Cammermeyer(8).

Comment. This communication reports the existence of what is believed to be a hitherto unidentified medullary structure which functions as a central emetic chemoreceptor apparatus. The problem of adventitious interruption of fiber pathways is invariably encountered in experiments involving lesions in the central nervous system. Pinschmidt(9) has shown that the emesis following parenteral digitoxin in the dog persists after denervation of the carotid and aortic chemoreceptor mechanisms. This finding was confirmed by us in one debuffered cat which gave a characteristic emetic response to lanatoside C injected intravenously. However, this animal failed to vomit after the CT zone was destroyed. Thus, destruction of the CT zone abolishes emesis by eliminating the selective site of attack of the cardiac glycosides and not by interrupting afferent pathways from extramedullary receptors.

The similarity in morphological characteristics between the CT zone and the medially situated area postrema raises the question whether these structures have similar functions. At the present time this question cannot be answered. Nevertheless, the fact remains that of these two regions, the CT zone was the only one found to be consistently destroyed in the operations resulting in abolition of emesis induced by intravenous digitalis. However, this does not exclude the possibility that the CT zone may have an additional role as the final common pathway for the transmission of activity from the contiguous vascular ependymal tissue (area postrema). Experimental study of this problem is underway.

From the anatomical part of this report it is clear that the CT zone borders upon, but is not a part of, the ala cinerea. It becomes questionable whether the ala cinerea is at all involved in the nervous regulation of vomiting. In this connection, there is the possibility of a species difference in medullary morphology between the dog and the cat. The experiments reported by Wang and Borison(1,2) were performed solely in the dog. The findings herein presented were obtained in cats, although some work was also done on dogs. The CT zone is not as readily identified in the dog as in the cat. This cellular formation is far less circumscribed in the dog and it is not improbable that scattered cells subserving the specialized emetic chemoreceptor function may actually exist in the canine ala cinerea. In the cat, however, the CT zone is seen as a clearcut morphological entity quite distinct from the dorsal vagal nuclei.

The discovery of a specialized chemoreceptor zone in the medulla oblongata makes it probable that other such structures exist in the central nervous system and in relation to the ependymal lining of the brain cavities. The demonstration of such areas in relation to the third ventricle, for example, would be of immense value in bridging large gaps in our present knowledge of hypothalamic and hypophyseal physiology. Furthermore, it remains to be determined whether the CT zone functions solely as an emetic chemoreceptor zone or whether it also has other functions analogous to those of the carotid and aortic bodies. In addition, the anatomical proximity of pressoreceptors and chemoreceptors in the carotid and the aortic regions suggests that pressoreceptors may exist in close association with the chemoreceptors of the CT zone. Such strategically located pressoreceptors could explain the emesis caused by elevated cerebrospinal fluid pressure. Further study is in progress to extend the present findings and to investigate other species.

Summary. 1. A hitherto unidentified structure in the medulla oblongata is described. It is an essentially non-neural zone situated between the ala cinerea and the vestibular complex and is contiguous medially with the area postrema which overlies the ala cinerea. The zone consists largely of fibroblasts and cells resembling protoplasmic astrocytes and astroblasts which have an abundance of vascular feet abutting on a dense network of blood vessels. Numerous nerve endings found in this zone are observed to connect with a loose fiber bundle which passes towards the nucleus of the fasciculus solitarius. 2. Experimental studies made with the ablation technic have yielded evidence that this structure is the anatomical framework of the medullary chemoreceptor trigger mechanism for the emetic center. Destruction of this chemoreceptor trigger zone in cats makes the animals refractory to the emetic effect of the intravenously administered cardiac glycosides—

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lanatoside C, scillaren A and ouabain—in doses which are known to be effective in intact animals.

The authors acknowledge the technical assistance of T. D. Harris. They are also indebted to Professors L. S. Goodman, W. S. Loewe and T. F. Dougherty for advice and encouragement.

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Morphologic Observations by Electron Microscopy of the Leon Strain of Poliomyelitis Virus. (18671)

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Electron microscopy studies conducted on the Brunhilde strain of poliomyelitis virus have been reported(1). The following study was undertaken with the Leon poliomyelitis strain. The Leon strain of poliomyelitis virus, which was isolated in Los Angeles in 1937 by Dr. John F. Kessel, was furnished by Dr. Jonas Salk and Major Byron Bennett of the Poliomyelitis Research Center, Municipal Hospital, University of Pittsburgh, Pittsburgh, Pa. The particular pool used, Leon Gen. XXI, has a 50% end point of $10^{-4.2}$ in rhesus monkeys by intracerebral inoculations.

Procedure. The rhesus monkeys used in the current study were healthy and were negative to the tuberculin test. They weighed approximately 5 lb each. Two monkeys were inoculated intracerebrally with 1.0 cc of a 1/50 dilution of poliomyelitis virus (Leon strain). Typical symptoms of poliomyelitis were noted on the 6th day after inoculation and the monkeys were sacrificed. The spinal cords were removed aseptically and pooled. Two monkeys were inoculated intracerebrally with 1.0 cc of a 1/50 dilution of normal monkey spinal cord which had been removed 8 days earlier, ground, and kept in the deep freeze at –40°C. No symptoms of central nervous system disorder were evidenced and both monkeys were sacrificed the 6th day post inoculation. The spinal cords were removed aseptically and pooled. The infected and normal pools were each ground with alundum and diluted to a 20% suspension with physiological saline. The 2 suspensions were then subjected to 5 minutes centrifugation at 1,000 rpm in an angle centrifuge. The supernatants were removed from the 2 specimens and filtered through a type ST size L3 Seitz filter. The filtrates from the 2 suspensions were then subjected to centrifugation under refrigeration in a Spinco ultracentrifuge for 3½ hours at 50,000 rpm. The temperature of the refrigerated outer jacket stayed constant at –14°C during the centrifuge run. After the 3½ hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1.0 cc of physiological saline. A small drop of each suspension was placed on several film supports. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium(2) at arc tangent 1/6 and examined under the RCA electron microscope, type EMU.

One monkey was inoculated intracerebrally with 0.4 cc of the concentrated normal material, and each of 2 monkeys was inoculated intracerebrally with 0.4 cc of the concentrated infected material. The monkeys receiving the concentrated infected material showed typical poliomyelitis symptoms after a period of 6 days and were sacrificed. Histological sections of the cords and brains showed typical pathological lesions of poliomyelitis. The monkey receiving the concentrated normal monkey cord appeared normal during a 30-day

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FIG. 1.
Poliomyelitis virus particles (Leon Strain) shadowed with chromium at arc tangent 1/6. Top x 75000; bottom both x 152000.

observation period.

Electron microscope examination of the concentrated virus suspension revealed virus-like particles as shown in Fig. 1. These particles were small and rod-shaped, ranging in width from 12-15 mμ. Upon examination of the concentrated suspension of the normal spinal cord no such virus particles were seen.

Summary. Studies by electron microscopy of the spinal cords of monkeys infected with poliomyelitis virus (Leon strain) showed rod-shaped, virus-like particles ranging in width from 12-15 mμ. These bodies could not be demonstrated in normal monkey cords.

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Comparison of Leukocyte Response to ACTH and Bacterial Pyrogen.* (18672)

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Bacterial polysaccharides, referred to as "pyrogens", induce characteristic alteration of the number of circulating white blood cells.

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† Hematology Research Foundation Fellow.

Following administration of such material, there occurs marked leukopenia succeeded by marked leukocytosis. These changes have been observed in a variety of species and are sufficiently characteristic of bacterial pyrogens to have prompted the suggestion that they be used as an alternative to the production of fever for the detection of such bacterial products in fluids to be used in human therapy. The alteration of the white blood cell count induced by pyrogen resembles that induced by stress, the administration of ACTH or the administration of adrenal steroids(1-9). In addition, purified pyrogens are reported to elicit tissue changes characteristic of the "alarm reaction"(10). Several observers have interpreted the eosinopenia produced in man by these bacterial pyrogens as evidence of specific pituitary-adrenal activity(11,12,13). However, in the rat, the typical lymphopenia induced by typhoid vaccine is not abolished by removal of the adrenals(14). In view of current interest in agents which are capable of initiating pituitary-adrenal discharge and in specific indicators of such discharge, it was thought worth-while to determine whether the leukocyte response to a purified pyrogen in the dog, which shows eosinopenia in response to ACTH, was or was not due to pituitary-adrenal activity.

This study has revealed that removal of the adrenal substantially reduces the eosinophile response to ACTH, whereas, the eosinophile response to pyrogen is not altered by this procedure.

Methods. Ten normal dogs were anesthetized with pentobarbital sodium. Blood samples were obtained from the femoral vein, a small amount of heparin was added and the total white cells as well as eosinophiles were enumerated in counting chambers. Eosinophiles were prepared for direct count by the eosine-acetone method of Dunger(15). After control counts had been obtained, 5 of these dogs were given 500 μg of either Baxter's P.S. pyromen‡ (prepared from *Pseudomonas aeruginosa*) or P.V. pyromen‡ (prepared from *Proteus vulgaris*) intravenously. The other 5 dogs received 10 mg of ACTH intravenously. The ACTH used was either Armour's 128-105 R§ or 72-50(C).§ The preparation labeled 128-105 R was reported to be 160% of the La-I-A standard. An additional 12 dogs were anesthetized with pentobarbital sodium and bilaterally adrenalectomized through a mid-abdominal incision. These animals were given pyrogen-free saline solution intravenously and 2-3 hours were allowed to elapse prior to collection of blood for control counts. Seven of these dogs were then injected with "pyromen" and 5 with ACTH in doses comparable to the control groups. Following the injection of ACTH or pyromen, blood samples were usually drawn at 5 min., 15 min., 30 min., and at hourly intervals for 6 hours. Total and eosinophile counts were performed in quadruplicate.

Results. Since no gross differences were ob-

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‡ Pyromen supplied through the courtesy of Dr. N. M. Nesset of Baxter Laboratories, Morton Grove, Ill.

§ ACTH preparations supplied through the courtesy of Dr. Irby Bunding of Armour and Co., Chicago.

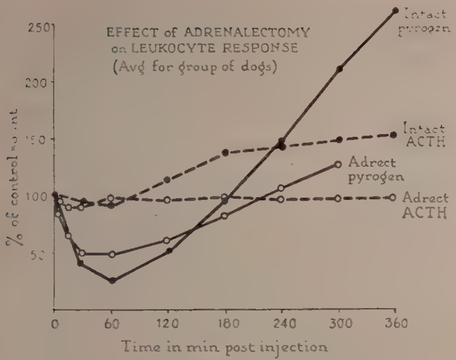

FIG. 1

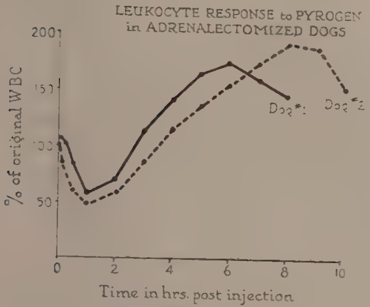

FIG. 2

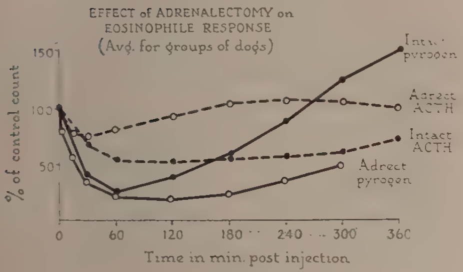

FIG. 3

served in the behavior of the two pyrogen preparations these animals are considered in one category. The same applies to the preparations of ACTH.

The control values for the various groups of animals are as follows: (1) white blood cells per cu mm, intact animals 8240 ± 4367, adrenalectomized animals 9773 ± 3172, eosinophiles per cu mm, intact animals 401 ± 324, adrenalectomized animals 519 ± 588. The response curve for each animal was smoothed by the procedure of running averages and counts were then expressed in terms of the percent of the control values for each animal. Fig. 1 is the average total white blood cell count for each group of animals expressed in terms of per cent of the control counts. It is apparent that, in the doses used, the leukopenia produced by ACTH is only very slight. That produced by pyrogenic material is more marked. Whereas, both pyrogen and ACTH produced some degree of leukocytosis during the period of observation, that produced by pyrogen is more marked.

Adrenalectomy neither abolished nor substantially modified the pyrogen induced leukopenia. ACTH induced leukocytosis was completely abolished by removal of the adrenals. However, the leukocytosis induced by pyrogen, subsequent to adrenalectomy, is somewhat less than that in the control group of animals. It should be indicated that adrenalectomized animals are quite susceptible to the lethal action of pyrogens and all of the present succumbed prior to the sixth hour of observation, at a time when leukocytosis is just beginning to be marked in the normal animal. Fig. 2 is an illustration of the fact that with lower doses of pyrogen (50 μg), marked leukocytosis can be induced in the adrenalectomized dog.

In Fig. 3 it may be seen that adrenalectomy substantially reduces the eosinopenia induced by ACTH. However, eosinopenia induced by pyrogen is, if anything, intensified by adrenalectomy.

The graphs presented are averages of the response of groups of animals. Hence, these composites are somewhat misleading due to the occurrence of maximum effects at varying periods. Thus a table has been prepared in which the magnitude of the maximum response has been reported for each animal without regard to the time of occurrence of this maximum response. From this table it may be observed that the average maximum depression of the W.B.C. count as produced by pyrogen was 76% before and 59% after adrenalectomy. No statistically significant difference exists between these two groups ($p = 0.06$). Correspondingly, the average reduction of circulating eosinophiles was 78% before and 86% after adrenalectomy. These

TABLE I. Extent of Maximum Depression of Circulating W.B.C.

	Pyrogen		ACTH	
	Adrenalectomized	Intact	Intact	Adrenalectomized
	% depression of total W.B.C.			
	—45	—83	— 3	— 7
	—62	—80	— 1	— 8
	—75	—75	—20	—14
	—74	—78	— 1	—13
	—65	—64	—55	—14
	—51			
	—38			
Avg	—58.6	—76.0	—16.0	—11.2
p		0.06	<0.01	>0.50
	% depression of eosinophiles.			
	— 68	—85	—78	—38
	— 79	—97	—53	—35
	— 82	—83	—83	—15
	— 99	—75	—48	—18
	—100	—50	—90	—35
	— 78			
	— 95			
Avg	—85.9	—78.0	—70.4	—28.2
p		>.40	>.50	<.01

groups are not significantly different (p = >0.40).

It may also be observed that the leukopenia induced by ACTH is significantly less than that produced by pyrogen (p = <0.01). However, the eosinopenia induced by ACTH is as great as that induced by pyrogen.

In contrast to the experiments with pyrogen, the maximum eosinopenia induced by ACTH is significantly reduced by adrenalectomy (p = < 0.01).

Discussion and conclusions. It is apparent from these studies that certain differences exist between the leukocyte response to ACTH and that to pyrogen. The typical eosinopenia induced by ACTH is mimicked by the injection of bacterial polysaccharides in the normal dog. However, adrenalectomy substantially reduces ACTH induced eosinopenia without modifying that induced by pyrogen. Thus, pyrogens are capable of producing these effects by mechanisms independent of pituitary-adrenal discharge. Such observations cast doubt upon the specificity of eosinopenia *per se* as an indicator of such discharge, and indicate that knowledge of the pyrogenicity of agents purported to induce adrenal discharge is desirable.

The failure of adrenalectomy, in these experiments, to completely abolish the eosinophile response to ACTH in the dog is in contrast to the findings of others(16), and raises the question as to whether the ACTH preparations employed here may not have been contaminated with bacterial polysaccharides. These preparations were tested for their fever producing potentialities in the dog and found to be free of such. However, it should be stated that in this animal moderate leukocyte responses can be obtained with non-pyrogenic doses of bacterial polysaccharides, and further, that in the anesthetized dog, maximum leukocyte responses without fever are the rule.

An additional difference between the leukocyte response to pyrogen and that to ACTH lies in the lack of substantial leukopenia following ACTH in doses sufficient to produce eosinopenia equal to that induced by pyrogen. This supports the contention that pyrogen is capable of its characteristic alteration of the number of circulating white blood cells by mechanisms other than adrenal discharge.

The present evidence suggests that the extent of the leukocytosis produced by pyrogen is somewhat less in the adrenalectomized than in the intact dog (Fig. 1). A similar observation has been made on the rat. The leukocytosis following typhoid vaccine appears to be less in this species following adrenalectomy(14). From this it might be inferred that leukocytosis following pyrogen is partially dependent upon adrenal stimulation. However, in our experience, as well as that of others(17), the adrenalectomized animal has a tremendously increased susceptibility to the lethal action of these bacterial products and these moribund preparations may be incapable, for non-specific reasons, of a sustained increase in leukocyte production. In partial support of this notion is the observation that smaller, and more readily tolerated, doses of pyrogen do give rise to sustained leukocytosis (Fig. 2).

Summary. The leukocyte response to bacterial pyrogen is superficially similar to that produced by ACTH. However, removal of

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the adrenals substantially modifies the response to ACTH in the dog, but is without effect on pyrogen induced leukocyte responses. It is concluded that pyrogen produces its characteristic changes in the W.B.C. count by mechanisms other than pituitary-adrenal discharge.

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Anti-Tubercular Activity *in vivo* of Nicotinaldehyde Thiosemicarbazone and Its Isomers. (18673)

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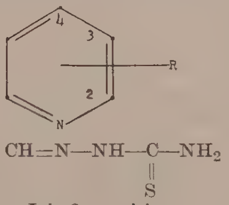
The anti-tubercular activity of 4-acetylaminobenzaldehyde thiosemicarbazone (Tibione) has been well established in experimental infections(1-5). The extensive chemical variations in the series of thiosemicarbazones has not yet resulted in chemically related compounds of appreciably higher activity(6-8), although the ethyl sulfonate of Tibione appeared somewhat superior to the parent substance in the studies by Hoggarth and Martin (4,8). These authors(4) also mention one heterocyclic compound of marked activity: quinoline-4-aldehyde thiosemicarbazone. The recent observations of Levaditi and his coworkers(9) according to which the thiosemicarbazone of nicotinaldehyde exerted a marked effect in experimental tuberculosis of mice seemed to offer a new approach to chemotherapeutic agents with anti-tubercular activity. Levaditi's findings are in agreement with the results of studies with compounds of this type carried out in our laboratories during the last year. This work was undertaken in connection with the studies of Chorine(10) and of McKenzie *et al.*(11) on the effect of niacinamide on experimental tuberculosis. While we were able to confirm the activity of this substance, we failed, as had McKenzie and his group(12) to find derivatives of niacinamide which were superior to the parent substance, until the thiosemicarbazone derivatives were synthesized in the Roche Chemical Laboratories. Of the numerous compounds synthesized by Drs. H. H. Fox, T. S. Gardner, F. A. Smith and E. Wenis and investigated during recent months, the biological properties of the following three substances will be described: 1. the thiosemicarbazone of picolinaldehyde (I); 2. the thiosemicarbazone of nicotinaldehyde (II), and 3. the thiosemicarbazone of isonicotinaldehyde (III). Tibione was used as the reference substance.



$$R = CH{=}N{-}NH{-}\underset{\underset{\displaystyle S}{\|}}{C}{-}NH_2$$

I in 2—position
II in 3— ”
III in 4— ”

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Materials and methods. Strain: The H37Rv strain of *M. tuberculosis* obtained through the courtesy of Dr. W. Steenken, Jr., of Trudeau Sanatorium on December 20, 1947 was used in all the experiments. *Culture*: The cultures were grown in 5.0 ml Dubos' medium containing Tween 80, but without albumin.

In vitro experiments. Serial dilutions of the compounds to be tested were made in 5.0 ml Dubos' medium containing Tween 80. Each test tube was inoculated with 0.05 ml of a 10^{-1} dilution of a 10- to 14-day-old culture of *M. tuberculosis* and incubated at 37°C. A final reading for the presence or absence of growth was taken at the end of 21 days. *In vivo experiments.* The technic of these experiments was similar to that recommended by Youmans(13). Adult albino mice of 18-20 g from one colony were used throughout the investigation. The mice were infected intravenously by route of the tail vein with 0.5 ml of a 10^{-1} dilution of a 7-10-day-old culture of *M. tuberculosis* in Dubos' medium which corresponds approximately to 100 minimal infective doses. Cultures of this age had a density of 65-70% light transmission determined by the lumetron photometer. The compounds to be tested were administered to the animals in the form of a medicated diet. This was accomplished by incorporating the desired concentration of the drug to be tested in powdered Rockland mouse food and allowing the animals to feed *ad libitum* from waste proof mouse feeders. At the end of 21 days, at which time all control animals were usually still alive, all mice were sacrificed and the lungs examined for the presence or absence of tuberculous lesions. The number of lesions present in lungs were usually graded in the following manner: 4+ = widespread foci in lungs; 3+ = numerous foci in lungs; 2+ = few foci in lungs; 1+ = 1-2 foci in lungs, and 0 = no observable foci. In the present note only the percentage of animals which were completely protected, *i.e.* absence of gross lesions, will be given. In order to characterize the response of this infection to the known anti-tubercular agents, the following data from numerous experiments are given: streptomycin, 50 mg/kg/day subcutaneously: 362 out of 453 mice protected = 80%; para-aminosalicylic acid, 2500 mg/kg/day by diet: 154 out of 159 mice protected = 96%; niacinamide, 2500 mg/kg/day by diet: 48 out of 50 mice protected = 96%. In addition to the intravenous infection the intranasal route leading to bronchogenic spreading has been used. The same culture dilution (10^{-1}) was selected as in the experiments with intravenous infection, since virulence tests had shown that the mice were equally susceptible to both routes of infection. The technic of the intranasal infection was the same as that described by Buck *et al.*(14) for the intranasal pneumococcus infection. Mice were infected, under light ether anesthesia, with four drops of a 10^{-1} dilution of a 7-10-day-old culture of *M. tuberculosis* H37Rv in Dubos' medium instilled by means of a 25 gauge needle from a 2.0 ml syringe. The mice were kept on medicated diet for 21 days after which time they were sacrificed and the lungs examined for lesions. These lesions were found to be not of the miliary type but of the confluent type characteristic for bronchogenic spreading. The lesions were graded as 4+ and 3+ where the entire lungs were affected in the sense of a tuberculous pneumonia while in the case of a lower degree of infection multiple smaller lesions of bronchogenic character were observed. In this note only the percentages of fully protected animals (absence of gross lesions) are given.

Experimental results. In vitro experiments. The soluble hydrochloride of nicotinaldehyde thiosemicarbazone was found to be bacteriostatic against *M. tuberculosis* H37Rv at a concentration of 1.6-3.2 mg % which agrees fairly well with the value of 6.0 mg % published by Levaditi *et al.*(9). The other 2 compounds were not tested because of their low solubility. Tibione was active at a concentration of 0.2 mg %.

Toxicity. Acute toxicity tests carried out by administering a single dose of the com-

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TABLE I. Anti-tubercular Activity of Thiosemicarbazones in Intravenous Infection of Mice with Strain H37Rv.

Infection: 0.5 ml of a 10^{-1} dilution of a 7-10-day-old culture in Dubos' medium.

Treatment: Medicated diet for a 21-day period.

Thiosemicarbazone	No. of mice	% medicated diet	Approx. dose: mg/kg	% protection
I	14	.02	50	0
II	48	.2	500	100
	59	.1	250	96.8
	10	.05	125	100
	18	.02	50	83.3
	20	.01	25	0
III	18	.2	500	100
	28	.1	250	90
	10	.05	125	80
	18	.02	50	80
	20	.01	25	0
Tibione	10	.2	500	100
	39	.1	250	97.5
	40	.05	125	95
	30	.02	50	50
	56	.01	25	1.8
Controls	141	—	—	0

pound by gavage gave an LD_{50} of 51.1 mg/kg for picolinaldehyde thiosemicarbazone, of 340.0 mg/kg for nicotinaldehyde thiosemicarbazone and of 931.0 mg/kg for isonicotinaldehyde thiosemicarbazone.

Since in the protection experiments with infected mice the drugs were given in the form of medicated diet over a 21-day period, the doses of the compounds are given in the tables as percent of drug in diet as well as in approximate mg drug/kg/day. This figure was calculated on the basis of the observation that mice normally consume 5 g of the medicated food per day so that 1% diet corresponds to 2500 mg/kg/day. The values thus calculated are generally but not always in reasonable agreement with the tolerated dose as evaluated by oral administration of a single dose by gavage. It happens sometimes that the animals tolerate more drug by medicated diet when the amount is consumed over a 24-hour period. In the experiments to be described this was the case with picolinaldehyde thiosemicarbazone.

In vivo experiments. The results of the experiments using the intravenous route of infection are given in Table I. It is evident from the data in this table that only the derivatives of nicotinaldehyde and isonicotinaldehyde thiosemicarbazone exerted an appreciable protective effect equal or even slightly better than that of Tibione. The thiosemicarbazone of picolinaldehyde was inactive.

The bronchogenic tuberculosis responded differently to anti-tubercular agents. The infection by the intranasal route was, as a rule, more resistant to anti-tubercular drugs than was the hematogenous infection. Para-aminosalicylic acid and niacinamide were of low activity even if high doses (2500 mg/kg/day by diet) were given. Our standard dose of streptomycin (50 mg/kg/day subcutaneously) protected not more than 10-20% of the animals, but full protection was observed with a daily dose of 150 mg/kg.

Tibione, on the other hand, and the thiosemicarbazones of nicotinaldehyde and isonicotinaldehyde were markedly effective in this type of infection. This is shown in Table II. From this table it may also be noted that nicotinaldehyde thiosemicarbazone and isonicotinaldehyde thiosemicarbazone were equally effective but 2 to 3 times less active than in the hematogenous infection. Tibione was less active than the heterocyclic thiosemicarbazones.

TABLE II. Anti-tubercular Activity of Thiosemicarbazones in Intranasal Infection of Mice.

Infection: 4 drops of a 10^{-1} dilution of a 7-10-day-old culture in Dubos' medium.

Treatment: Medicated diet for a 21-day period.

Thiosemicarbazone	No. of mice	% medicated diet	Approx. dose: mg/kg	% protection
I	8	.02	50	0
II	12	.2	500	100
	10	.1	250	90
	9	.04	100	55.5
	10	.02	50	0
III	10	.2	500	100
	20	.1	250	75
	10	.04	100	60
	10	.02	50	0
Tibione	10	1	2500	80
	16	.5	1250	62.5
	20	.2	500	30
Controls	38	—	—	2.7

Discussion. From the experiments described in the earlier part of this paper, it is evident that the thiosemicarbazones of nicotinaldehyde and of isonicotinaldehyde exerted a protective effect in the experimental hematogenous tuberculosis of mice. Levaditi's findings with the thiosemicarbazone of nicotinaldehyde were thus confirmed at least in the dosage of 50-100 mg/kg/day used by him. It seems, therefore, evident that the thiosemicarbazones of compounds which do not contain primary or secondary amino groups might exert an activity comparable or superior to Tibione.

Superior activity was particularly evident in the intranasal infection which was more resistant to such agents as para-amino-salicylic acid, niacinamide and also seemed to require considerably higher doses of Tibione than did the thiosemicarbazones of nicotinaldehyde and isonicotinaldehyde. No satisfactory explanation has as yet been found for this difference in activity.

Summary. (1) The anti-tubercular activity of the thiosemicarbazone of nicotinaldehyde in the intravenous infection of mice with *M. tuberculosis* as described by Levaditi was confirmed. (2) The thiosemicarbazone of isonicotinaldehyde was found as active as the thiosemicarbazone of nicotinaldehyde and the effect of both compounds was comparable to that of Tibione. Picolinaldehyde thiosemicarbazone was ineffective. (3) The thiosemicarbazones of nicotinaldehyde and isonicotinaldehyde also were found appreciably active in the therapeutically more resistant bronchogenic type of tuberculosis of mice produced by intranasal infection. Under these experimental conditions the compounds appeared superior to Tibione.

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Effect of Barbiturates on Oxidative Phosphorylation.* (18674)

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There is still much doubt as to the exact mechanism by which barbiturates produce their pharmacologic effects. Quastel and his associates(1) demonstrated that narcotics depress the respiration of brain tissue *in vitro* and postulated that the locus of the blocking action was at a flavoprotein step in the cytochrome chain. This supposition was further supported by the work of Grieg(2). However, both groups of workers used indirect means to demonstrate the postulated locus of action and did not specifically measure the step in question. Persky, *et al.*(3) have recently produced evidence which they interpret as showing that the site of action for the barbiturates is the dehydrogenase moiety of the pyruvic oxidase complex. Work in our laboratory has substantiated the overall inhibition of respiration in brain homogenates, but spectrophotometric measurements of DPN-cytochrome c reductase activity failed to show any unique sensitivity of this step to central nervous system depressants(4). Partial purification of the members of the cytochrome chain: DPN-cytochrome c reductase, cytochrome oxidase, and various dehydrogenases from brain, liver, and heart muscle did not disclose any enzyme which could be specifically inhibited by a reasonable

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† Fellow of the American Foundation for Pharmaceutical Education.

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concentration of the depressant drug(5). Furthermore, the recent work of Slater(6) subjects the earlier findings to reinterpretation. Using whole homogenates, no effect of narcotics on the efficiency of phosphorylations has been demonstrated(4,7), although such an effect could be shown with certain hypothermic drugs(8). The next logical step was the investigation of the effects of the narcotics on the phosphate:oxygen (P:O) ratios obtainable from the so-called particulate tissue preparations. This is a preliminary report on the effects of certain of the barbiturates on these preparations.

Experimental. Methods of tissue preparation and measurement of activity were based upon those now commonly used(9). A 10% homogenate of rat liver was prepared in 0.88 M sucrose with a Potter-Elvehjem homogenizer. The brain preparations were made in a similar manner except that 0.70 M sucrose, 0.20 M nicotinamide and 0.01 M sodium phosphate, pH 7.4, was used as a homogenizing medium. The homogenate was centrifuged (1500 x g for 3 minutes) and the resulting supernatant recentrifuged twice. The final supernatant was centrifuged (18,000 x g for 20 minutes), the residue resuspended in fresh homogenizing medium and recentrifuged at high speed as above. The final residue was resuspended in distilled water (2 ml/4 g tissue) and immediately pipetted into chilled Warburg vessels containing the materials as indicated in the legend of Fig. 1. All operations to this point were carried out at 5°C or below using cold reagents. The flasks were then placed on the manometers and equilibrated in the bath at 18°C for 10 minutes before starting to measure oxygen uptake. Enzymatic action was stopped by tipping in trichloracetic acid at the end of the desired period. Initial inorganic phosphate levels were obtained from vessels deproteinized when the oxygen uptake measurements were started (zero time). We were able to run experiments for as long as 70 minutes at 18°C without a decrease in the P:O ratios. Aliquots of the flask contents were analyzed for inorganic phosphate(10) and the net inorganic phosphate uptake determined by the difference between experimental flasks and the zero-time flasks. P:O ratios were calculated from these results and the concomitant oxygen uptake.

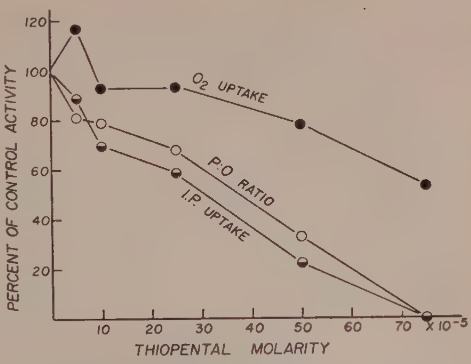

FIG. 1.

Effect of thiopental on oxidative phosphorylation in rat liver particulate preparations.

Vessel contents: 1.5×10^{-2} M Na-glycylglycine, pH 7.4, 2.0×10^{-2} M Na-phosphate, pH 7.4, 2.8×10^{-2} M glucose, 5.0×10^{-2} M KCl, 8.0×10^{-3} M $MgCl_2$, 2.5×10^{-3} M K-adenosine triphosphate, 8.3×10^{-6} M cytochrome c, 1.3×10^{-2} M Na-pyruvate, 4.3×10^{-3} M Na-fumarate, .1 ml yeast hexokinase (step 3a)(15), barbiturate concentration as indicated, .4 ml tissue preparation (see text), 1.2×10^{-2} M NaF, total volume 2 ml. Constituents added in the order given. .2 ml 50% trichloroacetic acid in sidearm. .2 ml 2 M KOH and filter paper in center well. Gas phase—air. Temperature—18°C. Duration of run 30, 40, or 60 min. (see text). Each point is the average of 3 separate experiments, percent activity for any individual vessel being calculated from its respective control. With regard to controls—from 13 separate experiments with a total of 24 individual vessels we obtained P:O ratios ranging from 2.29 to 3.12 with average of 2.65.

Results and discussion. We used thiopental, pentobarbital, amobarbital and phenobarbital and found that barbiturates are effective uncoupling agents. At a level of 1 x 10^{-4} M thiopental (Fig. 1) there is little or no decrease in the rate of respiration and yet the

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TABLE I. Effect of Barbiturates on Oxidative Phosphorylation in Particulate Tissue Preparations.*

	Rat liver									Rabbit brain		
	Pentobarbital			Amobarbital			Phenobarbital			Thiopental		
Drug molarity	O_2†	I.P.†	P:O‡	O_2	I.P.	P:O	O_2	I.P.	P:O	O_2	I.P.	P:O
Control	6.2, 5.2, 7.0	14.4, 12.8, 18.8	2.3, 2.4, 2.4	5.8, 4.4	16.4, 13.6	2.8, 3.1	7.1, 4.6	19.2, 11.2	2.7, 2.5	4.8, 3.8	9.6, 8.0	2.0, 2.1
,,	5.5	12.8	2.3	4.2, 4.5	11.6, 12.8	2.8, 2.8	6.2, 5.2	16.8, 12.8	2.7, 2.4	4.4, 3.2	8.8, 7.2	2.0, 2.3
1×10^{-5}	6.9	16.0	2.3									
5	7.2, 5.4	17.6, 13.6	2.4, 2.5	4.7	12.8	2.7	4.4	11.6	2.7	5.5, 3.2	8.0, 6.4	1.6, 2.0
1×10^{-4}	5.5, 5.4, 5.3	13.6, 13.6, 12.0	2.5, 2.5, 2.2	4.2	11.2	2.7	3.9	11.6	2.9	4.3, 3.2	8.0, 6.0	1.8, 1.9
2.5	6.7, 4.6	14.4, 10.6	2.1, 2.3	4.2	10.0	2.4	4.3	10.4	2.4	4.6, 3.1	5.6, 5.6	1.4, 1.8
5	2.9, 1.5	1.6, 0.8	0.6, 0.5	1.7	1.2	0.7	4.0	8.8	2.2	3.7, 3.2	2.4, 2.2	0.6, 0.8
7.5	1.4, 0.4	+2.4, +3.2	0.0, 0.0	0.6	+2.4	0.0	3.9	9.6	2.1	3.6, 1.8	0.0, 0.0	0.0, 0.0
1×10^{-3}	0.9, 0.4, 0.0	+4.0, +4.0, +2.4	0.0, 0.0, 0.0	0.0, 0.2	0.0 +2.4	0.0, 0.0	5.7, 3.5	9.2, 8.0	1.6, 2.2	2.9, 2.2	0.0, 0.0	0.0, 0.0

* Conditions and vessel contents same as indicated for Fig. 1, except in the case of the brain system which contained in additon 5.0×10^{-4} M diphosphopyridine nucleotide.

† Oxygen and inorganic phosphate uptakes expressed as microatoms.

‡ Phosphate:oxygen ratios calculated as described in text.

P:O ratio is depressed. With only moderately decreased (20-40%) respiration at concentrations of 5.0 to 7.5 x 10^{-4} M thiopental, the most potent of the barbiturates, almost completely inhibits phosphate uptake. For a lesser effect greater amounts (1.0 x 10^{-3} M) of phenobarbital were required (Table I). These effects were observed both in rat liver and in rabbit brain particulate preparations (Table I), but in the latter the P:O ratios were somewhat lower and consequently the results not optimal.

Like Persky *et al.*(3) we have tested the effect of the four barbiturates on the yeast hexokinase "trapping" system and find no inhibition at levels as high as 5 x 10^{-3} M. Hence, the uncoupling effect must be due to a depression of some prior transphosphorylation.

The chief objection to attributing the *in vivo* effects of narcotics to their inhibition of respiration detected *in vitro* is the relatively high concentrations of drug required in the latter case to produce effects of significant magnitude(3,12). From the minimum surgical anesthetic doses given by Gruhzit, *et al.*(13) for rabbits, assuming the distribution of an intravenous dose of barbiturate to be equal throughout the body, we have calculated the corresponding molar concentration in the tissue. For thiopental, amobarbital and pentobarbital these concentrations are 8.0 x 10^{-5} M, 2.5 x 10^{-4} M, and 2.0 x 10^{-4} M respectively. There is no great disparity between these values and those which gave a significant depression of the P:O ratio, namely: 1.0 x 10^{-4} M, 5.0 x 10^{-4} M and 5.0 x 10^{-4} M.

The uncoupling effects reported here are not nearly as striking as those which are observed with dinitrophenol and gramicidin(14) possibly because there is more overlap between the

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concentrations of barbiturates necessary to inhibit phosphorylation and those necessary to inhibit respiration.

McElroy(12) proposed that narcotics act not by merely depressing the respiratory rate of nervous tissue but by inhibiting some energy-yielding process, notably phosphate bond turnover, thereby depressing or abolishing neuronal activity. A recent paper by Westfall(15) indicates that small doses of barbiturates produce a small increase in the oxygen consumption of rat cerebral slices. Our data show a similar effect with particulate preparations (Fig. 1). Thus, barbiturates are no exception to the suggestion of Lardy and Elvehjem(16) that compounds which speed up the metabolic rate at the same time decrease the available energy by allowing respiration to take place without phosphorylation.

Further evidence that the mere depression of the respiration of the brain is inadequate to explain the pharmacologic effects of these compounds is shown by comparing the metabolic deficiencies of the brain in hypoglycemic coma and in thiopental anesthesia(17). In the former, metabolic rate and nervous activity fall equally; while in the latter, the fall in metabolic rate is small when the narcosis is deep. Larrabee, *et al.*(18) have also shown in studies on excised superior cervical ganglia that nervous activity may be depressed without a proportionate fall in oxygen consumption. All of which indicates that barbiturates may depress nervous activity *in vivo* by uncoupling oxidation from phosphorylation.

Summary. It has been shown that thiopental, pentobarbital and amobarbital lower the phosphate : oxygen uptake ratio obtained in particulate tissue preparations from liver and brain respiring on a pyruvate substrate. This depression is obtained at concentrations of the drugs which have only a slight effect on the oxygen uptake of the preparations and which approximate those which are necessary *in vivo* to produce surgical anesthesia. It is postulated that such an uncoupling of phosphorylation from oxidation may be one of the ways in which barbiturates act to produce anesthesia.

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Chemical Composition of Bovine Spermatozoa. (18675)

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Information on the chemical composition of spermatozoa is fundamental to a better understanding of the functional significance of the male gamete in the fertilization process as well as its contribution to subsequent developmental processes. There have been in the past several investigations dealing with various aspects of this problem. The work of Kossel(1) on the protamines of fish sperm in his researches dealing with the structure of basic proteins is an example. More recent-

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ly, Zittle and O'Dell(2) determined the methionine content of bovine spermatozoa and Sarkar *et al.*(3) reported values on several amino acids found in sperm of the same species.

Data are presented here on 17 amino acids present in bovine spermatozoa as well as on the total nitrogen, total lipid, total phosphorus, and ash content.

Materials. Preparation of spermatozoa. Semen samples were collected at weekly intervals from 7 bulls used for artificial insemination investigations at Iowa State College. Microscopic examination of the semen samples used in this work indicated that little, if any, contaminating material such as tissue debris or leucocytes was present. Each ejaculate ranging from 3 to 7 ml was diluted with two volumes of buffered 0.9 per cent NaCl (pH 6.8) and centrifuged. The supernatant fluid was aspirated and the spermatozoa were resuspended in buffered saline and allowed to stand 10-15 minutes before centrifuging. The spermatozoa were washed 8 to 10 times in a similar manner with buffered saline and finally washed once with distilled water. The samples of the washed spermatozoa were dried at 105°C, composited, and stored for chemical analyses and microbiological assays. Approximately 700 mg of material were prepared for use in this work.

Methods of analysis. Total nitrogen was determined by the micro-Kjeldahl method, total lipid by ether extraction of moisture-free material, and ash by ignition after adding sulphuric acid. Phosphorus was determined gravimetrically according to a procedure described by Elek(4) and Elek and Hill(5). The amino acids were determined by microbiological assays and the response of the test organism was determined titrimetrically. Acid hydrolysis was used in the preparation of dried bovine spermatozoa for the amino acid assays except for tyrosine and tryptophan where alkaline hydrolysis was employed. Leucine, isoleucine, and valine were determined by use of *Lactobacillus arabinosus* 17-5 by a modification of the procedure developed by Shankman(6). The media as used in these determinations contained all of the recognized nonessential amino acids and 0.6% ammonium chloride, 2% dextrose, and 1.2% sodium acetate. Tryptophan was determined in a similar medium but ammonium chloride was omitted. Glutamic acid was assayed according to the method of Dunn *et al.*(7) and ammonium chloride was added to the medium. *Leuconostoc mesenteroides* P-60 was used in determining lysine, histidine and phenylalanine employing methods described by Dunn *et al.*(8-11). Glycine was determined according to Shankman *et al.* (12,13) using the same organism. An unpublished procedure with *L. mesenteroides* P-60 by Shankman was used for the assay of serine, proline, and aspartic acid. The amino acid level in these media was 1.33 times that employed in the previously indicated lysine assay. Incubation time was 3 days. Hydroxyproline and norvaline were omitted from the media used in determining these amino acids. Cystine was assayed with *L. mesenteroides* P-60 according to Camien and Dunn (14). Hydrolyses with 1:1 hydrochloric-formic acid under reflux and with 10% hydrochloric acid in a sealed tube gave identical

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data for this amino acid.

Lactobacillus fermenti 36 was used for the threonine and methionine assays and the procedure of Dunn *et al.*(15-17) was followed. Threonine was also assayed with *Streptococcus faecalis* according to Stokes *et al.*(18). Arginine and tyrosine were determined with *Lactobacillus casei* according to Shankman *et al.*(19) and this medium was modified to contain 2% dextrose, 1.2% sodium acetate, and all the amino acids indicated except hydroxyproline, norleucine, and norvaline. Two assays, at 2 different dilutions of hydrolyzate, were made for each amino acid. A control assay on casein hydrolyzate was also made with each amino acid determination and the entire assay was repeated if the results obtained did not agree with accepted values in the literature for casein. A common complete vitamin mixture was used for all assays. Furthermore, in evaluating the analytical data presented here, it is necessary to stress that amino acid assays on whole sperm do not differentiate between free amino acids which may be present in the cell and those constituting protein molecules.

Results. The total nitrogen content of bovine spermatozoa was found to be 16.7% on a moisture- and lipid-free basis. Analyses of dry sperm showed a total lipid content of 1.2%, ash 1.1%, and total phosphorus 2.3%. The results of the amino acid assays are presented in Table I.

TABLE I. Amino Acid Composition of Bovine Spermatozoa. Results expressed in % on a moisture, ash, and lipid-free basis.

Amino acid	Assay 1 %	Assay 2 %	Avg %
Arginine	15.4	14.6	15.0
Aspartic acid	5.0	5.0	5.0
Cystine	2.8	2.7	2.8
Glutamic acid	6.2	6.5	6.4
Glycine	3.5	3.6	3.6
Histidine	1.7	1.7	1.7
Isoleucine	2.4	2.5	2.5
Leucine	4.6	4.4	4.5
Lysine	4.3	4.5	4.4
Methionine	1.5	1.4	1.5
Phenylalanine	2.6	2.7	2.7
Proline	3.1	3.1	3.1
Serine	4.4	4.5	4.5
Threonine	3.3	3.1	3.2
Tryptophan	1.0	1.0	1.0
Tyrosine	4.6	3.9	4.3
Valine	3.2	3.3	3.3

Discussion. Dried bovine spermatozoa are high in total nitrogen as indicated by the data presented here. The average value of 16.7% is somewhat lower than the value obtained by Sarker *et al.*(3). Arginine is the most abundant amino acid present in the sperm cell and the value of 15.0% obtained in this work is much lower than the 25.47% reported by Sarkar *et al.*(3). Furthermore, the histidine, lysine, tryptophan, phenylalanine, leucine, isoleucine, and glutamic acid data presented in Table I are lower than those presented by Sarkar *et al.*(3). However, there is good agreement between both sets of data for methionine, threonine, and valine. Zittle and O'Dell(2) have reported the methionine content of bovine sperm as 1.92% on a moisture- and lipid-free basis.

The bovine spermatozoon may be characterized as a cell with a small amount of cytoplasm and a large nucleus and, as a result, this cell is relatively high in nucleoprotein. It is of interest that tryptophan occurs in small amounts in this cell as indicated by the data in Table I.

It was found by Melampy(20) in a cytochemical study of the amino acids of the chicken erythrocyte that the tryptophan content of the nuclei was low. Mirsky and Ris (21) in their work on the composition of chromosomes have found a small amount of tryptophan in the histones of fowl erythrocytes. Methionine and histidine are also present in relatively low concentrations in the bovine sperm cell.

Summary. The chemical composition of

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bovine spermatozoa has been determined. Data are presented including total N, total lipid, ash, and total phosphorus. Seventeen amino acids were determined by microbiological assays.

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One Stage Method of Hepatectomy in the Dog.* (18676)

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Complete removal of the liver from dogs has been achieved by various methods(1-3). The following new, one-stage method has given satisfactory results to the authors and can be executed without great difficulty. The abdomen having been widely opened, and the incision kept apart with a self retaining retractor, the gastro-hepatic omentum is cut, after the portal vein has been freed from all other structures. The portal vein is cut against the liver, introduced into a small metallic tube according to Queirolo's method(4) and implanted sideways into the vena cava above the renal veins. The liver is then severed from the abdominal wall. When this is done, a strong thread is placed around the vena cava against the diaphragm in order to prevent the reflux of the blood from the heart. The flow of the blood to the liver has been previously stopped by 2 clamps placed one on the vena cava and the other on the portal vein upstream from the portal-cava fistula. Then a veinous graft which is to replace the intra-hepatic part of the vena cava is put into position. The graft is the intra-thoracic part of the vena cava of another dog used in a previous operation; it has been kept at 0°C in Tyrode solution to which penicillin and a sulfamide have been added. After the vena cava of the dog has been cut transversely under the liver and against it, the inferior part of the graft is inserted into the caudal segment of the vein and fixed above the portal-cava fistula. The other end is introduced into the intra-hepatic part of the vena cava and pushed upwards until it finds its place between the top edge of the liver and the diaphragm. The ligature lying behind the liver is then firmly tied on it. The clamps placed on the portal vein and the vena cava being taken away, one proceeds to the extirpation of the liver after having split the intra-hepatic part of the vena cava from the lower end to the top.

Dogs which have undergone this operation, performed with an aseptic technic, survived for 12-14 hours. Death occurs from circulatory collapse. To restore the vascular tonus, we resorted to the use of a hydrosoluble derivative of desoxycorticosterone added in sufficient quantity to the glucose solution, which is injected, drop by drop, into a vein. The survival of 3 dogs which received this treatment, was respectively 16, 26 and 31 hours.

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Effect of Protein Depletion on Response to Water, Sodium Chloride and Pituitrin. (18677)

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The following study was undertaken to demonstrate the effect of protein depletion in otherwise healthy animals upon the excretion of water and sodium chloride. Progressive depletion of serum proteins was accomplished by dietary restriction combined with plasmapheresis. The responses of the normal and depleted animals to water, sodium chloride and pituitrin were studied. A brief account of these experiments follows.

Methods. Water balance and metabolic studies were accomplished during an experimental period lasting 90 days, equally divided into the control period and the period of protein depletion, each of 6 weeks duration.

Four healthy, episiotomized female dogs were placed on a purified diet containing 25% casein and providing 40 calories per lb. body weight (Table I). During the depletion period the casein content was reduced to 4% in the diet of 3 dogs, the fourth remaining as a control. In addition plasmapheresis was performed 3 to 5 times per week on the 3 dogs undergoing depletion. Twelve to 15 cc of blood per kg were withdrawn each time, the heparinized plasma was discarded and the washed red cells resuspended in 0.85% NaCl and injected into a vein. Plasma proteins (albumin and globulin) were determined by the micro-Kjeldahl procedure twice a week using 27% sodium sulfate for their separation.† The glomerular filtration rate as measured by the clearance of creatinine (0.2 g/kg subcutaneously)(1) and extracellular fluid volume by the sodium thiocyanate method(2) were determined weekly. A 5-hour period for the study of water excretion appeared most suitable for these experiments. Following the administration of 50 cc of water per kilo by stomach tube, the time period to excrete 50% of the water load was measured before and after 0.01, 0.025, and 0.05 units/kg of pituitrin administered subcutaneously. (All pituitrin used in these experiments was from the same lot).‡ Chloride excretion was(7) measured before and after administration of 50 cc of 0.85% NaCl per kg by stomach tube by following the 24-hour excretion for 3 subsequent days. Chloride excretion and response to water and pituitrin were studied on alternate weeks. The chloride content of diet remained constant. Dog A served as a control, dogs B, C, and D were placed on a protein deficient diet and subjected to plasmapheresis 3 to 5 times weekly after completion of 6 weeks of control observations.

TABLE I. Composition of Diets.

	Control 25% protein diet	Low 4% protein diet
Casein	25%	4%
Sugar	40	61
Lard	20	20
Yeast	5	5
Salt mixture U.S.P. No. 1	5	5
Cellurex	5	5
Percomorph	5 drops/lb	5 drops/lb

Dogs were fed 40 calories per lb body wt. Weight was maintained at approximately the same level throughout this experiment.

Experimental results. 1. During the 6-week period of protein depletion, total serum proteins declined from control values ranging from 7.00-5.50 g % to 4.5-3.3 g % (Fig. 1). Serum albumin showed the greatest depression during the first 4 weeks; however, the globulin levels gradually became lower over the 6-week

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*The authors wish to thank Dr. Ida Kraus and Miss Margaret Griesser for technical assistance.

† Electrophoretic studies carried out on samples of normal and protein depleted animals indicated that 27% sodium sulfate gave only slightly higher albumin values than were obtained by electrophoresis.

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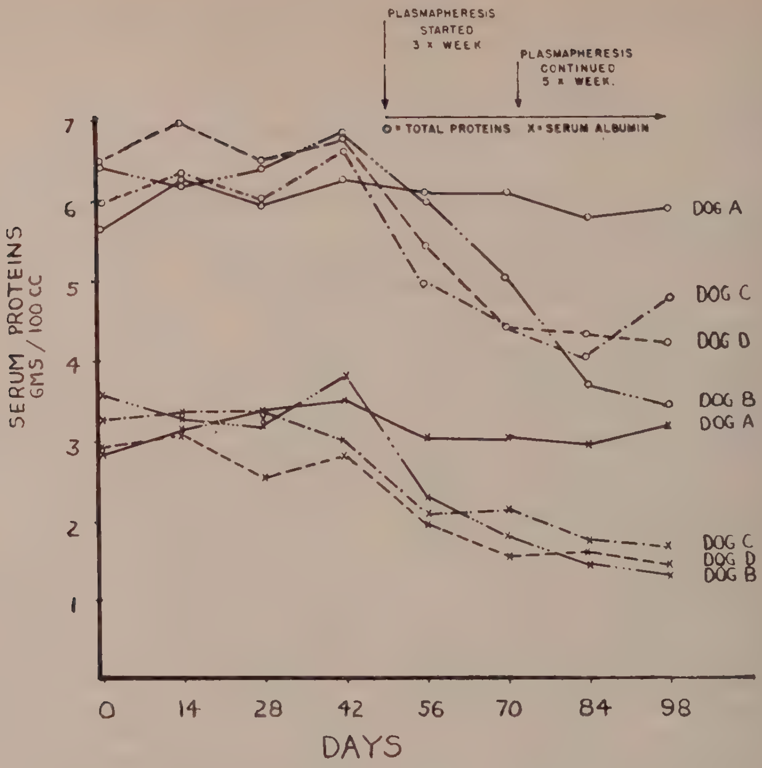

FIG. 1.
Effect of protein depletion upon the total serum proteins and serum albumin fraction.

period. Albumin values declined from a control range of 3.9-2.7 g % to 1.92-1.31 g %.

2. The time required to excrete 50% of a water load of 50 cc/kg increased slightly in all dogs. The results, however, are not statistically significant except for dog B (Table II).

3. Sixty to 70% of the test dose of sodium chloride was excreted within the first 24 hours. No significant change was noted in the rate of chloride excretion by the protein depleted dogs following ingestion of 50 cc/kg of 0.85% NaCl.

4. As serum proteins progressively decreased, the extracellular fluid volume in terms of per cent body weight increased, although the differences in values are not statistically significant. In all dogs the thiocyanate space attained its highest level when the serum proteins were lowest (Table III).

5. As can be seen in Table III, the glomerular filtration rate decreased in all dogs maintained on a low protein diet. Lowest values were recorded when the serum proteins reached their maximum depression.

6. The degree of urinary suppression produced by the administration of a dose of pitu-

TABLE II. Effect of Serum Protein Depletion on Time Required to Excrete 50% of Water Load.

	Before protein depletion time in hr (mean)	After protein depletion* time in hr (mean)	t-ratio
Dog A (control)	2.48	2.36	0.53
B	1.51	10.93	3.55
C	2.50	4.61	2.04
D	2.21	3.10	1.05

* 5-6 wk of protein depletion.

TABLE III. Effect of Serum Protein Depletion on Extracellular Fluid Volume and Glomerular Filtration Rate.

	Extracellular fluid volume* Thiocyanate space as % body wt		Glomerular filtration rate Creatinine clearance cc/min.	
	Control period	Protein depletion*	Control period	Protein depletion†
Dog A (control)	28.7	27.6	54.6	51.2
B	29.5	33.9	79.7	39.8
C	29.4	29.9	53.6	35.3
D	36.1	38.3	57.5	28.4

* Mean of 3 determinations.
† 5-6 wk of protein depletion.

TABLE IV. Effect of Serum Protein Depletion on Pituitrin Sensitivity in Terms of Relative Potency Units.

	Protein before depletion	After depletion	t-ratio
Dog A (control)	52	54	0.106
B	65	369	5.24
C	142	168	0.50
D	121	146	1.04

itrin is expressed in terms of relative potency units derived from a dose-response curve of mean values obtained during the control period (Table IV).

Analysis of the data obtained from a series of experiments varying the dose of pituitrin form 0.01 to 0.05 unit per kilo body weight revealed considerable variations in degree of urinary suppression between dogs. The control animal, dog A, responded to pitruitin in a fairly consistent manner. Dog B exhibited a marked increase in sensitivity to pituitrin as the serum proteins declined. The other two dogs likewise showed increasing sensitivity to pituitrin but to a lesser degree. The change was only statistically significant for dog B.

Discussion. These results show that hypoproteinemic dogs require a slightly longer period to excrete a water load than during the control period of study. Although the extracellular fluid volume increased slightly in all protein deficient dogs, gross edema was not observed. Ingestion of excess sodium chloride did not lead to edema formation and, according to the test employed, no gross impairment in chloride excretion was demonstrated in the hypoproteinemic animal.

Protein deficient dogs showed an increased suppression of urine to a given dose of pituitrin as compared to observations during the control period. Some reduction in glomerular filtration in dogs maintained on a diet low in protein has been demonstrated previously by Shannon(3). The increased sensitivity to pituitrin may be related to the reduction in glomerular filtration although the animal that was most sensitive to pituitrin did not have a lower plasma albumin or glomerular filtration rate than the other depleted animals. Wakim *et al.*(4) have stated that pituitrin decreased the renal blood flow and the rate of urine formation. Leslie and Ralli(5) have observed that the urine of rats on a high fat low protein diet exhibited marked antidiuretic properties. Impaired hepatic function produced by the protein deficient diet(6) may reduce pituitrin inactivation by this organ.

Summary. 1. The time required to excrete 50% of an ingested water load was increased in 3 protein-depleted dogs. 2. The glomerular filtration rate was reduced in all protein-depleted dogs. 3. The urinary suppression produced by pituitrin was increased in the protein-depleted dogs.

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Effect of Alkalis on Conjugation of Sulfonamides with Glucuronic and Acetic Acid.* (18678)

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The glucuronide of sulfonamides is more soluble(1) and bacteriostatic(2) than the acetylated derivative. These considerations make it worthwhile to determine the effect of various substances given to increase the solubility of sulfonamides in urine upon their tendency to form conjugates of acetic or glucuronic acid.

The present study was undertaken to obtain comparative data on the effects of sodium bicarbonate, sodium citrate, sodium lactate, and urea on the blood concentration, urinary excretion, and the extent of conjugation with glucuronic and acetic acid, of ingested sulfadiazine and sulfamerazine in human beings.

Methods. These studies were carried out on 2 hospitalized patients with well controlled diabetes mellitus. One patient was a 24-year-old white female requiring 64 units of insulin daily. Moderate glycosuria† was present part of the time. The other was a 70-year-old white female receiving 20 units of insulin daily who had no glycosuria. Both were on a constant, quantitative diet throughout the experimental period. After several days without sulfonamide medication, in order to establish control glucuronic acid values, the sulfonamide was given orally every 6 hours for 3 days. The adjuvant drug was given with the sulfonamide and continued for several days after the sulfonamide was stopped so that it could be determined whether the changes in glucuronic acid values were due to the sulfonamide or the adjuvant. Sulfonamide was not given again until no sulfonamide or only traces were present in the urine and blood, and another control glucuronic acid value was obtained before giving another adjuvant. Sodium bicarbonate was given as the powder in water, sodium citrate as effervescent tablets,‡ sodium lactate as an effervescent mixture of sodium bicarbonate and lactic acid, and urea as the U.S.P. drug. Urea was given in 15 g doses every 6 hours in fruit juice which was part of the constant, quantitative diet. Alkali dosages were equivalent to 24 g of sodium bicarbonate daily. Venous blood samples were drawn each morning just prior to medications. Twenty-four urines were collected under toluene. Sulfonamide determinations were made by the method of Bratton and Marshall(3) using 1:40 blood filtrates and the Evelyn photolometer. Urea nitrogen was determined by the method of Gentzkow(4). Glucuronic acid was estimated by the method of Maughn, Evelyn, and Browne(5) with slight modification. The acetylated sulfonamide fraction is calculated as the difference between "free" and total sulfonamide as determined by the method of Bratton and Marshall(3). The concurrent increase over control levels of urinary glucuronic acid when sulfonamide is excreted in the urine is assumed to be due to glucuronic acid conjugated in equimolar combinations with sulfonamide. The sulfonamide conjugated with glucuronic acid is included in the free fraction since sulfapyridine glucuronic acid(2) was diazotized without hydrolysis. The sulfonamide excreted in an uncombined or unconjugated form is calculated as the difference between the "free" form and that combined with glucuronic acid.

Results. There was no general correlation

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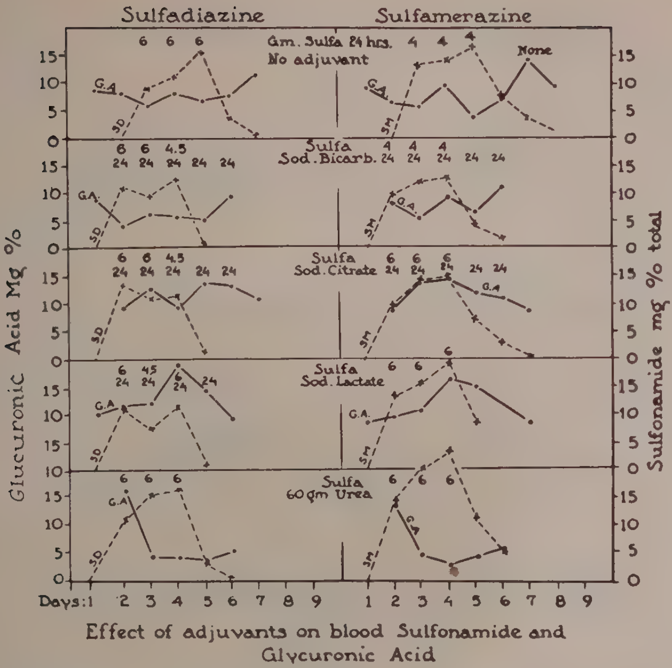

Fig. 1.

Effect of adjuvants on blood concentration of glucuronic acid and total sulfonamide.

of glucuronic acid and sulfonamide blood levels (Fig. 1). Blood glucuronic acid concentrations were above control levels when sulfadiazine or sulfamerazine was given with sodium citrate or sodium lactate adjuvants, and unchanged or below control levels when given with urea, sodium bicarbonate, or with no adjuvant. Average blood glucuronic acid concentrations are shown in Table I.

The average acetylated fraction in the blood was 5% with sulfadiazine and 7.7% with sulfamerazine. No significant differences due to the various adjuvants were demonstrated.

TABLE I. Average Blood Glucuronic Acid Concentrations.

	Patient A mg %	Patient B mg %
Controls*	8.9	8.1
No adjuvant	7.0	9.4
Sodium bicarbonate	5.6	8.1
” citrate	11.3	11.7
” lactate	14.4	13.0
Urea	7.0	6.9

* There were 18 controls for sulfadiazine (Patient A) and 5 controls for sulfamerazine (Patient B).

Fig. 2 shows 24-hour urinary contents of free and total sulfonamide and glucuronic acid with the administration of various adjuvants. An increase of glucuronic acid is shown in urines containing either sulfadiazine or sulfamerazine both with and without adjuvants. All values are of single experiments except the curves with sodium citrate and urea given with sulfadiazine which are averages of 2 curves each.

Fig. 3 shows the percentage of total urinary sulfonamide conjugated with glucuronic acid, acetic acid, and uncombined. Total conjugation (acetylated plus glucuronates), was not altered by any of the adjuvants, with the exception of sodium bicarbonate on the patient given sulfadiazine. No explanation can be offered for this exception. Conjugation with acetic acid was less after the administration of adjuvant alkalis than after urea or no ad-

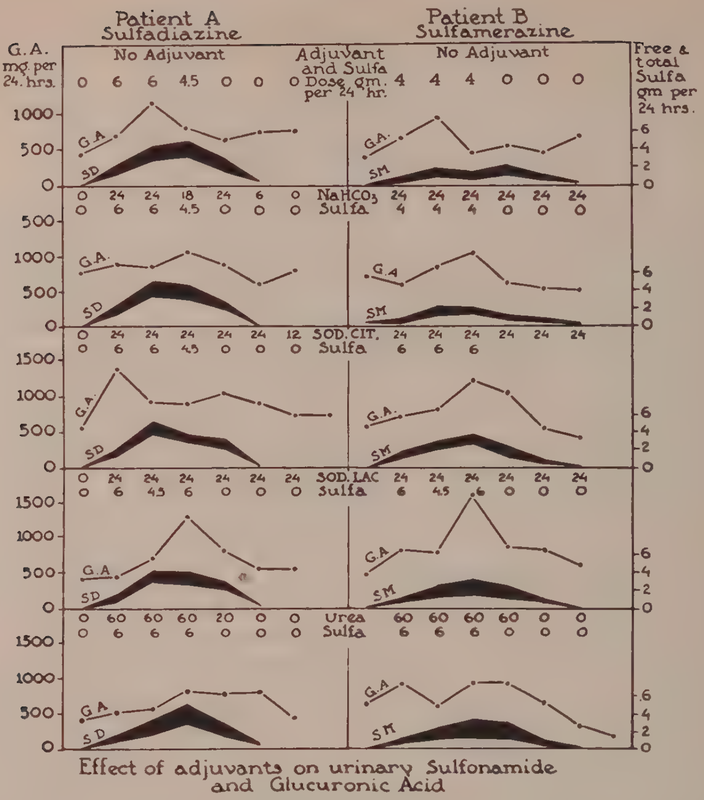

FIG. 2.

Effect of adjuvants on urinary excretion of glucuronic acid and sulfonamides. Top border of black band represents total sulfonamide and bottom border represents free sulfonamide.

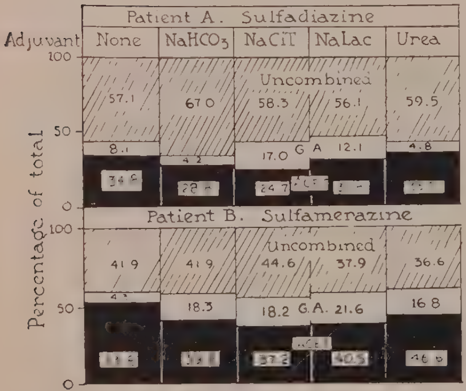

FIG. 3.

Effect of adjuvants on percentage of urinary sulfonamide uncombined or conjugated with glucuronic acid or acetic acid.

juvant was given. Conjugation with glucuronic acid was greater after alkalis were administered as adjuvants with sulfamerazine, and after sodium citrate and sodium lactate were given with sulfadiazine. Urea had no significant effect on the conjugation of either sulfonamide with acetic or glucuronic acid.

Fig. 4 shows the blood urea nitrogen concentration and urine urea nitrogen content after the administration of 60 g of urea daily with sulfonamides. Seventy-two per cent of the ingested urea was recovered in the urine of patient A and 55% in patient B. Blood and urine urea nitrogen values had returned almost to control levels 24 hours after stopping the administration of urea.

Discussion. Slightly lower blood sulfona-

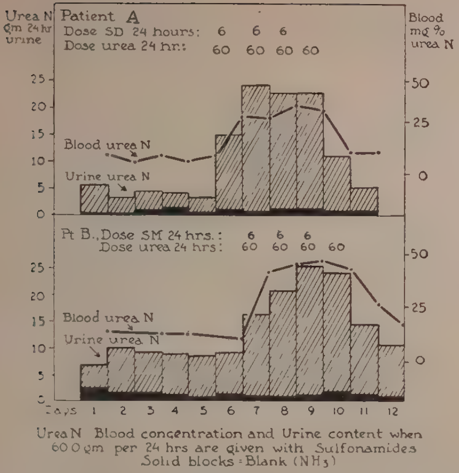

FIG. 4.

Urea nitrogen blood concentration and urine content when 60 g of urea is given daily with sulfonamides.

mide concentrations after alkali administration have been reported(6,7) and may be explained on the basis of accelerated urinary excretion due to decreased tubular reabsorption of the glucuronide derivative. Increased blood glucuronic acid after sulfadiazine therapy has been reported(8). It has been reported(9) that sulfamerazine, but not sulfadiazine, increases glucuronic acid in human urine. Fig. 2 and 3 show increased urinary glucuronic acid after both sulfamerazine and sulfadiazine. The effect of sodium salts on glucuronide formation may be related to the extra base available for excretion, to alkalosis or the effect of the anion. The fact that sodium bicarbonate had less effect on the blood and urine glucuronic acid content than sodium citrate or lactate suggests that these compounds may have a specific effect on glucuronide formation. Martin(10) reported that lactic acid increased urinary glucuronic acid in the rat. Lipschitz and Bueding found lactate to increase conjugated glucuronides *in vitro*(11). Quick(12) reported that in the dog decreased urinary excretion of benzoic acid conjugated with glucuronic acid when lactic acid was given with benzoic acid and no effect when sodium bicarbonate was substituted for lactic acid. Reduction in the percentage of acetylated sulfadiazine in the urine of human beings receiving sodium lactate has been reported(13). Sodium bicarbonate as well as citrate and lactate salts reduced the percentage of acetylated sulfonamide in urine.

Urea apparently has no effect on the blood concentration, excretion or conjugation of sulfonamides. Clinically, urea reduces the incidence of crystalluria but not as effectively as 24 g of sodium bicarbonate per day(6). However, alkalis may be contraindicated under certain circumstances.

Summary. These studies indicate that alkalis, particularly sodium citrate and sodium lactate, may increase the conjugation of sulfamerazine and sulfadiazine with glucuronic acid and decrease conjugation with acetic acid in human subjects.

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Field Studies on Carbon Dioxide as a Possible Host Simulant to Mosquitoes.* (18679)

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It has been known for a number of years that certain materials provoke a positive chemotropic response in mosquitoes(1), yet little application has been made of this knowledge in developing new technics for the collecting or study of mosquitoes in nature. Rudolfs(2) demonstrated that mosquitoes were strongly "activated" by an atmospheric increase of carbon dioxide and ammonia, and he believed that these stimuli played an important role in attracting mosquitoes to their hosts. Subsequently, studies have been reported in which this knowledge was applied by using dry ice as an adjuvant with mosquito light traps(3-5). Although the number of mosquitoes collected was increased, knowledge of mosquito behavior was not amplified, nor was the technic practical for general use.

These observations led in the present study to the use of carbon dioxide alone as a mosquito attractant to determine the applicability of such a stimulus in studying the apparent feeding preferences of different mosquito species. Data from preliminary field investigations on the role of mosquitoes in the epidemiology of virus encephalitis in Kern County, California, during 1950 indicate that a valuable technic for such studies may be developed. A recent report(6) on field studies of the various factors which may serve to attract mosquitoes to man substantiates the importance of carbon dioxide as one of the stimuli.

Methods. Two stable traps of the design described by Bates(7) were employed as the standard apparatus to collect the mosquitoes. In preliminary trials, the 2 traps were comparable in parallel trials using dry ice baits. Under these conditions, variations in the number of mosquitoes collected in the 2 traps were those expected by chance. Dry ice was the source of carbon dioxide. A 7 to 9 lb. piece of dry ice, wrapped in 2 thicknesses of heavy paper with a single longitudinal slit through both layers, was placed in a 6-gallon can with a screen top. This can was suspended in the trap at the level of the ingress baffle. For animal bait a single calf or 3 to 5 chickens were used in each test. Each trap was baited shortly before or at sunset. In comparative tests with dry ice and animal baits, the two traps were baited at the same time and were run in the same area, usually separated by a distance of 10 to 20 yards. Some dry ice remained the following morning. On the morning following trials, all mosquitoes were removed from the traps and transported to the

TABLE I. Species and Number of Female Mosquitoes Collected in 49 Trials with Dry Ice Bait.

		No. collected	
Mosquito	Total	Mean per trial	Median per trial
Culex tarsalis	9036	184	178
Culex quinquefasciatus	578	12	1
Aedes dorsalis	175	4	1
Aedes nigromaculis	4477	91	20
Others*	11	0.3	—
Total	14277	291	220

* Species included *Anopheles franciscanus*, *Culex erythrothorax*.

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† The author is indebted to R. O. Hayes and A. Rudnick for their assistance in this study.

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TABLE II. Species and Number of Female Mosquitoes Collected in 8 Trials, Comparing Dry Ice and Calf Baits.

	Species and No. of mosquitoes collected									
	C. tarsalis		*C. quinquefasciatus*		*A. dorsalis*		*A. nigromaculis*		All species	
Bait	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial
Dry ice	1291	161	62	8	4	.5	764	96	2121	265
Calf	879	110	43	5	4	.5	400	50	1326	166

TABLE III. Species and Number of Female Mosquitoes Collected in 7 Trials, Comparing Dry Ice and Chicken Baits.

	Species and No. of mosquitoes collected									
	C. tarsalis		*C. quinquefasciatus*		*A. dorsalis*		*A. nigromaculis*		All species	
Bait	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial	Total	Mean per trial
Dry ice	2350	336	195	28	19	3	335	48	2799	400
Chicken	780	111	120	17	2	0.3	21	3	923	132

laboratory for identification and counting.

Results. In 49 trials employing dry ice as an attractant, a total of 14,277 female mosquitoes of 6 species were collected (Table I).

In 8 trials to compare the attractiveness of dry ice and calf baits, the 4 principal mosquito species of the area were collected. The dry ice bait consistently attracted more mosquitoes than did the animal bait (Table II).

In 7 trials to compare the values of dry ice and chicken baits, dry ice again consistently attracted larger numbers (Table III).

On 3 occasions small numbers of female mosquitoes (2, 6, and 13) were found in unbaited traps.

Male mosquitoes were seldom found in unbaited traps or in traps baited with dry ice or animals. With dry ice, males of the following species were collected: *Culex tarsalis*, 3; *Culex quinquefasciatus*, 2; and *Aedes nigromaculis*, one. With calf bait, 2 male *C. tarsalis* were collected, and with chicken bait, one male *C. tarsalis*.

Discussion. The results of these preliminary trials indicate that carbon dioxide alone can be used to attract female mosquitoes. This method of collecting samples of mosquitoes has been of value in areas where electricity or experimental animals are not available. Mosquito samples collected in this fashion were satisfactory in disease vector studies. Because of the open construction of the trap, the mosquitoes are not asphyxiated by the carbon dioxide, and there is no reason to suspect that disease agents such as viruses are affected by it in the living vector. Another definite advantage over collections from animal-baited traps is that few of the mosquitoes are freshly engorged with blood meals which may include disease agents or immune bodies, either of which would be misleading in interpretation of field data.

These findings seem to warrant further studies in which the volume of carbon dioxide released is carefully controlled. It would seem appropriate in such studies to use the average amount of carbon dioxide produced by bovine, human, avian, and amphibian hosts. This would represent a wide host range and markedly different volumes of carbon dioxide and possibly would clarify the unexplained host preference of different mosquito species.

If a carbon dioxide chemotropism is a major attracting force, one might hypothesize that the chemotropism of different mosquito species differs. *Culex apicalis*, for example, might be extremely sensitive to a small amount of carbon dioxide, such as is given off by amphibians, but be repelled by the higher concentrations excreted by larger vertebrates. Other species such as *Culex tarsalis* and *Culex quinquefasciatus*, known to have a wide host range from small to large vertebrates(8,9), could be expected to exhibit a broad chemotropic sensitivity. Many species of *Aedes, Anopheles,* and other genera which feed predominantly on large mammals(10,11) would respond only to relatively high concentrations of carbon dioxide.

The observation that carbon dioxide specifically attracted female mosquitoes may be further evidence that this chemotropism is closely allied to search for a blood source.

The significance of host body temperature as an attracting force appears to be eliminated. However, dry ice materially reduces temperature and increases relative humidity in the immediate area of the tests. Therefore, in future studies, some other source of gas such as a portable carbon dioxide generator should be used to obviate these objections.

It would be of interest to test the attractiveness of carbon dioxide in areas inhabited by species such as *Aedes varipalpus, Aedes triseriatus,* or *Aedes albopictus.* Males of these species are frequently observed in the vicinity of animal hosts supposedly for the purpose of coming in proximity to females for mating. Demonstration that these males are attracted by carbon dioxide could serve as further evidence of the specificity of this chemotropism.

Summary. Carbon dioxide alone was a mosquito attractant in disease vector studies. Large numbers of female mosquitoes of several species were attracted into a trap baited with carbon dioxide. It consistently attracted more mosquitoes than calf or chicken baits. This chemotropism may be a major factor in controlling host selection by various species of mosquitoes.

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Effect of ACTH and Cortisone on Homogenous Kidney Transplants. (18680)

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The reported efficacy of ACTH and cortisone in states of hypersensitivity(1) and in antigen-antibody reactions(2) has led to a reconsideration of the possibility of influencing the biological incompatibility of homogenous renal transplants(3-5) by the use of these agents.

Method. Sterile technic was employed. A donor kidney was obtained from a healthy heparinized dog. The pedicle was cleared of extravascular tissue and its vessels anastomosed to the common carotid artery and external jugular vein of the recipient dog by direct end-to-end union with continuous everting sutures, or by the use of vitallium tubes(6). Anastomosis by direct suture required 20-25 minutes; by the use of vitallium tubes, less than 10 minutes. After fixing the kidney to prevent rotation, the ureter was brought out through a stab wound in the opposite side of the neck with care to avoid kinking. Urine collection was facilitated by

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TABLE I. Comparative Analytic Data from Bladder and Transplant Urines.

Exp. No.	Anastomosis	Therapy	Day	Specific gravity B*	Specific gravity T*	pH B	pH T	Alb. B	Alb. T	Cells B	Cells T	Urea (mg %) B	Urea (mg %) T	Chlorides (mg %) B	Chlorides (mg %) T	Eosinophil count Pre-therapy	Eosinophil count During therapy
I	Direct	ACTH	1	1.014	1.016	7	6	0	0	—	—	418	202.4				
			2	1.020	1.020	6.5	5	0	+	—	—	750	693				
			5	1.012	1.010	7	7	0	+	—	RBC	73.7	114.4	750	665		
II	,,	ACTH	1	1.015	1.015	6	6	0	0	—	—	1364	299	150	315	1940	141
			2	1.020	1.022	6	7	0	0	—	—	1170	169	50	45		100
			4	1.026	1.024	5	6.5	0	+	—	RBC	1056	209	65	125		194
III	,,	Cortisone	1	1.014	1.012	7	6.5	0	+	—	RBC	—	—	—	—	1600	150
IV	Vit. tube	,,	1	1.012	1.016	7	6.5	0	0	—	—	—	—	—	—	2000	100
			2	1.014	1.012	7	7	0	0	—	—	325	410	80	75		134
V	,, ,,	,,	1	1.022	1.020	6	7	0	0	—	RBC	—	—	—	—	1800	120

* B—Bladder urine. T—Transplant urine.

placing into the exteriorized ureter a polyethylene catheter attached to a sterile condom from which urine samples were taken for analysis. Five animals were so prepared. All received 300,000 units of aqueous procaine penicillin once daily. Cortisone (75 mg) was given intramuscularly to three dogs three times a day. The other 2 dogs received 100 mg of ACTH intramuscularly daily for several days and then 50 mg daily. Eosinophil counts(7) were done once daily. Daily analytic results of urine obtained from the transplant were compared with those of urine taken simultaneously from the bladder by suprapubic aspiration with a sterile needle. When the transplanted kidney ceased excreting urine, it was removed for microscopic examination.

Results. Adequate response to the hormones was indicated by the marked eosinopenia observed in all instances (Table I).

All the transplanted kidneys functioned immediately upon completion of the anastomosis. A daily intravenous injection of 200 cc of 25% glucose in distilled water, or of 300-500 cc of 5% glucose in distilled water produced a marked diuresis from the transplanted kidney as well as from the recipient's own kidneys. During the first hour after each infusion 50-125 cc of urine was excreted by the transplanted kidney. Comparison of the urine analyses showed close correlation of specific gravity and pH, but not of urea or chloride content.

The appearance of albumin, and later of blood, in the urine from the transplanted ureter signified impending functional collapse of the transplant. When the transplant failed to respond to the infusion it was considered functionless, and was then removed. Functional survival of the transplants averaged 60 hours (range 24 to 132 hours). This was no better than that reported by other investigators without the use of ACTH or cortisone (3,4). At the time of excision of the transplant, the anastomoses were patent and free of clot or thrombus formation.

The excised transplanted kidney was always swollen. There was edema of the ureters,

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and the perirenal fat was indurated. There was no evidence of sepsis.

Sections of the transplant were fixed immediately after removal. In three of the transplants microscopy showed various stages of tubular degeneration. One showed definite necrosis of tubular epithelium and tubular casts. The others showed slight glomerular and peri-glomerular lymphocytic infiltration, but no tubular degeneration. The perirenal fat showed lymphocytic infiltration and red cell extravasation. The pelves showed subacute inflammation.

Conclusions. The length of survival or function of homogenous kidney transplants is not improved by the administration of ACTH or cortisone.

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Effect of Proteinuria on Localization of Radiomercury in Rat Kidney.*

(18681)

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It was shown by the use of radiomercury-labeled diuretics that these compounds are selectively concentrated in the kidneys of patients(1) and in the kidneys of normal dogs(2). In our previous work(3) it was found that proteinuria, produced by the intraperitoneal administration of human albumin to rats prior to the intravenous administration of a mercurial diuretic, sharply reduced renal toxicity. This effect was attributed to an inhibition of mercurial absorption when the tubules were saturated with protein. At the same time it was found that the simultaneous administration of human albumin and the mercurial diuretic resulted in enhanced toxicity. The latter effect was attributed to more rapid mercurial absorption, concomitant with rapid absorption of protein during the tubular loading phase.

The present study was undertaken in order to determine whether proteinuria was associated with an actual reduction in the amount of mercury that localized in the kidney, thus diminishing the dose to which the renal epithelium was subjected. The use of radioactive mercury with the technic of radioautography offered the possibility of a simple, semi-quantitative evaluation of the localization phenomenon.

Methods and materials. This study was performed with 24 female rats of the Slonaker strain, each weighing about 150 g, with little variation. The radiomercury used was obtained from the Oak Ridge National Laboratory and was certified to be more than 99% Hg^{203}. The radiomercury was administered in the form of mercuric chloride, 10 mg Hg per ml distilled water, at pH 5.4. In the control group of 12 animals, each received 3 intraperitoneal injections of 16 ml 0.85% sodium chloride solution, the first at 9:30 A.M., and the second at 4:30 P.M. on the first day, and the third at 9:30 A.M. on the second day. In the experimental group, each animal received similar injections on the same schedule, but each injection was composed of 6% bovine albumin in 0.85% sodium chloride solution. From previous experiments(4) it is

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† Fellow of the John Simon Guggenheim Memorial Foundation.

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known that bovine albumin, like human albumin, produces a maximal degree of proteinuria at the interval selected after such intraperitoneal administration. Immediately after the third intraperitoneal injection, each animal received 0.10 ml of the radiomercuric chloride solution by intravenous injection. This dose contained 1.0 mg Hg, with a radioactivity of 50 μc. Each group of animals was placed in a separate cage, with wood shavings in the bottom to absorb the urine and feces. Stock diet and water were given *ad libitum*. The animals were not disturbed until death, or for 24 hours, when the survivors were killed by ether anesthetization and exsanguination from the abdominal aorta. At autopsy, the kidneys were removed, decapsulated, sagittally sectioned, and dropped into 10% formalin in 0.85% sodium chloride solution. After fixation, the tissues were embedded in paraffin and sections were cut at a thickness of 8 μ. These sections were used to make radioautographs on Eastman Kodak NTB Nuclear Track Plates. Exposures were made on 10 μ and 25 μ emulsions with 8 to 15 million beta particles per cm^2. In order to eliminate errors arising from differences in emulsion sensitivity, development, etc., some control sections were mounted on each NTB plate with an equal number of experimental sections. After completion of the exposure, the plates were developed and the paraffin sections were stained with hematoxylin and eosin for microscopic examination.

Results. The control animals that received saline solution by intraperitoneal injection all died before 24 hours had passed after administration of the radiomercuric chloride. At autopsy, the kidneys appeared to be of normal size with a color that was slightly darker than the normal. Only 2 of the animals that received bovine albumin injections died during the 24-hour period. With 2 exceptions, the surviving animals appeared to be well at the time they were killed. At autopsy, the kidneys of all these animals were enlarged and pale, similar to the kidneys of animals that receive such injections of albumin alone, but somewhat larger.

A portable Geiger-Müller counter was used to survey the wood shavings in each cage which had been used to absorb the urine and feces during the period after injection of the radiomercuric chloride. The shavings used for the control animals had roughly half the radioactivity shown by the other shavings. This was considered to indicate a significant difference in the amount of radioactive material excreted.

Inspection of the radioautographs revealed a gross and consistent difference in the radioactivity of the sections. The control animals had a much higher amount of radioactivity in the cortical substance; this appeared to be about twice as much as in the experimental animals. (Fig. 1). In addition, the control animals did not show any radioactivity in the medulla, even in the plates which were overexposed. On the contrary, about one-half of the experimental animals showed radioactivity that extended into the medulla and outlined the medullary pyramids. These observations were considered to indicate that, 24 hours after injection, under the conditions of this experiment, mercury was still being excreted by the experimental animals, whereas in the control animals such excretion had ceased before they died.

Discussion. If it is assumed that all of the radiomercury injected was localized in the kidney and that none was excreted, then in a 150-g rat with kidneys weighing about 1100 mg, the kidneys would have received less than 200 roentgen-equivalents of β-radiation in the 24-hour period. Since the kidney is relatively insensitive to radiation damage(5), this factor can be ignored in the interpretation of the tracer results. The LD_{50} for total body irradiation of the rat is 500 to 600 r, and, under the assumptions made, the whole body received less than 5 r during the 24-hour period, so this factor may also be ignored in consideration of the tracer effects.

Because radiomercury-labeled diuretic was not available to us, we were obliged to use an inorganic salt for this experiment. The difference in mortality between the control group and the experimental group demonstrates that proteinuria has the same effect upon the renal

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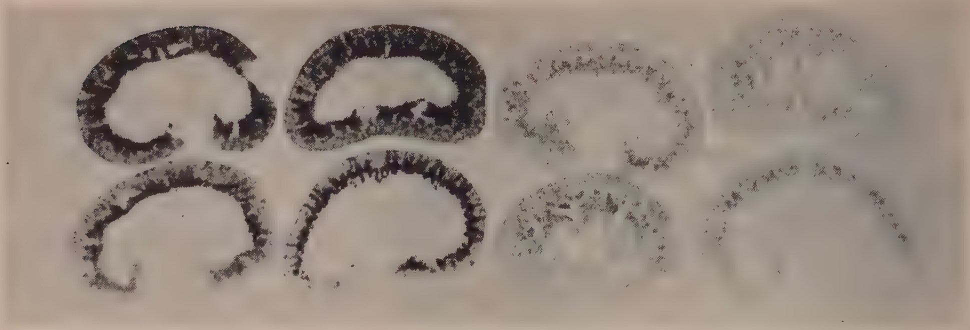

FIG. 1.

Radioautograph of kidneys, 24 hr after administration of radiomercuric chloride. Left, control group. Right, experimental (proteinuric) group. Eastman Kodak NTB plate, 10 μ emulsion, 10 million counts per cm^2, magnification about 3×.

toxicity of inorganic mercuric chloride as it has upon the mercurial diuretic.

In our previous work(3) we were unable to demonstrate that mercurial diuretic was bound by serum protein. However, since that time Milnor(6) has succeeded in such a demonstration. It is well known that mercuric ion is bound by protein, and this experiment shows that proteinuria prevents the mercuric ion from localizing in the kidney. It would seem reasonable to suppose that, in view of Milnor's work, an organic mercurial diuretic would behave in the same way.

Summary. When radioactive mercuric chloride is administered to animals rendered proteinuric by the administration of bovine albumin, less radiomercury is localized in the renal cortex than in that of the control animals. Diminished renal toxicity is associated with a diminished localization of radiomercury in the kidney.

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Sensitivity of Mice to Histamine During Respiratory Infection by *Hemophilus pertussis.* (18682)

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It was first shown by Parfentjev and Goodline(1) that pertussis vaccines can markedly increase the sensitivity of mice to histamine. Pittman(2) has reported that vaccines of relatively low toxicity may show a direct correlation in ability to induce sensitization and protection in mice; a toxic vaccine, however, induced better protection than sensitization. During her study, it was observed that a higher percentage of vaccinated mice which had survived an intracerebral inoculation of *H. pertussis,* were sensitive to histamine than other mice correspondingly vaccinated but not challenged(3). This suggested that the living bacteria might also be capable of inducing a sensitivity and that this property might have some bearing on the clinical manifestations of the disease in man. The intracerebral route of infection in the mouse is highly artificial

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TABLE I. Mortality and Time of Death in Mice Infected Intranasally.

Exp. No.	No. of bacteria inoculated, ×1000	Period of observation, day	Deaths			
			D/n	%	Avg	±S.E.
9	10000	50	27/ 80	33.75	16.1	±1.01
	1000		11/ 80	13.75	23.5	±2.06
14	10000	45	49/110	44.5	17.7	±0.85
	1000		26/ 90	29.0	19.3	±0.98
	100		11/ 50	22.0	21.1	±2.73
26	50	28	18/178	10.1	20.8	±1.02

D/n = Deaths over No. inj.

and unless vaccinated mice do not survive. By the intranasal route(4), however, a high degree of infectivity with low mortality can be induced. This infection also has the advantage that, in some respects, it resembles the human infection. In the present paper, there will be reported some observations on the histamine sensitivity and the presence of gross lesions and of *H. pertussis* in the lungs of mice inoculated intranasally with *H. pertussis* and on the resistance of recovered mice to an intracerebral inoculation of *H. pertussis*.

Materials and methods. A culture of *H. pertussis* (No. 1/92) which had a high virulence for mice by the intracerebral route, was grown for 20 hours on a Bordet-Gengou agar slant, suspended in a solution of 1.0% protolysate (a tryptic digest of casein) and 0.5% sodium chloride and then instilled in the nares of anesthetized female mice, in a volume of 0.05 ml. Only female mice were used because both normal and vaccinated female mice are more sensitive to histamine than male mice(5). At intervals during the course of the infection the sensitivity to histamine diphosphate was determined by injecting intraperitoneally 100 mg per kg in 0.2 ml distilled water. The lungs of all mice that died or had survived for 2 hours after the injection, were examined for gross lesions and cultured on Bordet-Gengou agar slants. Some experiments carried normal mice for histamine controls, these mice were not sensitive to the dose of histamine used and are not reported in the results. The LD_{50} of histamine for the normal female mouse is around 1900 mg per kg(5).

Results. Mortality and time of death. In Table I are given the times and percentages of death of the mice in relation to the inocula of culture observed in 3 experiments. In the first two, mice were withdrawn at varying intervals for histamine sensitivity determinations. In the former, withdrawals were started earlier than in the latter. This may explain the lower mortality obtained in it. Experiment 26 was observed for specific deaths for only 28 days. It may be seen that the number and time of death were related to the amount of culture injected but even with the largest dose more than 50% survived, while the average time of death for the four doses ranged from 16.1 to 23.5 days. The earliest death was on 5th day and the latest on 41st day. An infection with this duration and relatively low mortality afforded an excellent opportunity to observe the development and duration of histamine sensitization in mice infected with *H. pertussis*.

Histamine sensitization. In the experiment reported in Table II, two groups of mice of 100 each were injected with 10- and 1 million bacteria, respectively, then at intervals between 5 and 50 days, mice in groups of 10 or more were tested for sensitivity to histamine. The larger dose of culture induced, by the end of 10 days, a high degree of sensitivity, which persisted for many days; at the end of 50 days, 33% of the remaining mice were still sensitive. The LD_{50} of histamine at 15 days was 47 mg per kg. Following the inoculation of the smaller dose, the rise in sensitization was slower. From this experiment and No. 14 reported in the next section, it appears that by 45 or 50 days when the mice had started to recover from the infection that sensitization was decreasing. At the end of 5 days, there

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TABLE II. Histamine Sensitization in Relation to Pathology and Infection. (Exp. 9).

No. of bacteria inoculated, ×1000	Histamine diphosphate				Gross lung pathology			*H. pertussis* recovered		
	After culture, days	Amount, mg/kg	Deaths							
			D/n	%	P/D	P/S	Total %	C/D	C/S	Total %
10000	5	100	6/10	60	6/6	4/4	100	4/6*	3/4	70*
	10	100	10/10	100	10/10		100	10/10		100
	15†	100	5/5	100	5/5		100	4/5		95
		50	6/10	60	6/6	4/4		6/6	4/4	
		25	0/5	0		5/5			5/5	
	20	100	8/10	80	8/8	1/2	90	6/8	1/2	70
	30	100	10/13	77	9/10	2/3	85	1/10	1/3	15
	50	100	3/9	33	2/3	3/6	56	0/3	0/6	0
1000	5	100	1/10	10	1/1	6/9	70	1/1	4/9	50
	10	100	5/10	50	5/5	0/5	50	5/5	2/5	70
	15	100	7/10	70	7/7	1/3	80	6/7	1/3	70
	20	100	5/10	50	5/5	1/5	60	4/5	0/5	40
	30	100	6/16	38	4/6	1/10	32	0/6	0/10	0
	50	100	11/22	50	10/11‡	1/11	50	0/11	0/11	0

* Two cultures were overgrown with a contaminant.

† LD_{50} of histamine at 15 days = 47 mg/kg, 2σ limits 37.7 and 58.6 mg/kg. D = deaths; S = survival; n = No. inj.; P = lung lesions; C = positive cultures.

‡ In some the lungs appeared grey, unlike those observed in other mice.

was a higher incidence of lung involvement than sensitization, thereafter the presence of gross lesions and sensitization was closely correlated. Through the first 15 days the recovery of *H. pertussis* was correlated with the pathology. At 30 days and later, bacteria were recovered from very few mice. In Table III is given a summary of observations made in 4 experiments on the incidence of lung lesions and positive *H. pertussis* cultures in relation to death from histaminic shock. Of the mice that died, both lung pathology and positive cultures were obtained from 58.8%, while 84.8% showed pathology and from 64.8% positive cultures were obtained. The incidence of each manifestation was significantly lower in the mice that survived. If it is taken into consideration that in the mice which survived, the highest incidence of lung lesions was in those tested early, and that in the mice which died the highest incidence of negative cultures was in those tested late, then we do indeed have a close correlation between histamine sensitization and the *H. pertussis* infection in the mice.

TABLE III. Correlation of Lung Pathology and Lung Cultures with Histamine Sensitization of Mice from 4 Experiments.

Lung		Response to histamine			
		Died, 182		Survived, 116	
Pathology	Culture	No.	%	No.	%
+		154	84.6	34	29.3
	+	118	64.8	22	19.0
—		28	15.3	82	70.7
	—	64	35.1	94	81.0
+	+	107	58.8	13	11.2
+	—	47	25.8	21	18.1
—	+	11	6.0	9	7.8
—	—	17	9.3	73	62.9

Resistance of recovered mice. In several experiments, the mice that had survived the inoculation of culture intranasally were given an intracerebral injection of a culture of another strain of *H. pertussis* (No. 18323). Most of the mice were resistant. The results of one experiment (No. 14) are given in Table IV. Following the intranasal injection, the course of the infection and sensitivity had been similar to that reported for experiment 9 in Table II. Mice from 3 groups, which had been given 10,000-, 1,000-, and 100 thousand bacteria, respectively, were tested for histamine sensitivity at 15, 30, and 45 days. The percentages of shock deaths of mice from the respective groups at 15 days were 80, 90, and 60; at 30 days, 100, 82, and 100; and at 45 days, 20, 41, and 40. A high percentage of those that died showed lung lesions. At 15 days the percentage of positive lung cultures

TABLE IV. Resistance of Mice to Intracerebral Infection After Intranasal Inoculation.

No. of bacteria inoculated		No. survivals/No. inj. intracer.	*H. pertussis* recovered from†		
			Brain		Lung
Intranasally, ×1000	Intracerebrally,* ×1000		Survivals	Dead	Dead
10000	100	12/12	1		
	20	10/12	1	1	0
	4	11/12	1	0	1
1000	100	13/15	0	2	1
	20	15/15	1		
	4	13/14	1	1	0
100	100	8/12	1	2	0
	20	6/10	0	2	0
	4	8/10	0		
	Total/n	96/112	6/96	8/12	2/12
	%	85.7	6.25	66.7	16.7
		Controls			
0	100	1/10	1		
	20	0/10			
	4	4/10	1		
	2	3/10	0		
	.4	4/10	0		
	.08	10/10	1		

* 45 days after intranasal inoculation. LD$_{50}$ of culture = 550 bacteria.

† Cannibalism prevented the culturing of 4 mice. Cultures were not made of the lungs of mice that survived or of the brain of the control mice that died.

was also high; at 45 days, in the first group, all cultures were negative, but in the other two, 18 and 20%, respectively, were positive. This incidence is higher than was obtained in experiment 9.

At the time of the last sensitivity test the remaining mice in each group were injected intracerebrally with 3 doses of culture, respectively, as indicated in Table IV. They were observed for 14 days; those that had symptoms of brain involvement at the end of this time were counted among the dead mice. There was no apparent difference in the response to the different intracerebral doses in the mice that had been given the same intranasal inoculum. It appears, however, that better protection was obtained in the mice that had received the two larger intranasal inocula. Eighty-six per cent of all mice survived; from 6 of these, although the mice showed no symptoms, positive brain cultures were obtained.

Discussion. Mice with an induced *H. pertussis* respiratory infection developed a high degree of sensitivity to histamine which was comparable to that induced by 300- to 1000 million bacteria of good mouse-protective pertussis vaccines (non-precipitated) (2). It differed from the latter in that the maximum degree of sensitivity was not reached for 10 or 15 days, it persisted much longer and it was closely correlated with the presence of lung lesions. The development of sensitivity appears to have some relation to the multiplication of the bacteria in the lungs and not to the lesions *per se* as they were present before a high degree of sensitivity developed but sensitivity persisted longer than the bacteria. With vaccine, the peak of sensitivity is reached in 4 or 5 days, followed by a rapid drop.

The course of the infection and the histamine sensitivity in the mouse strikingly parallels the onset and duration of paroxysmal coughing in the child. In the child, there is an incubation period of 7 to 10 days, a localization of the bacteria in the respiratory tract and lungs, and a paroxysmal cough of long duration which persists after *H. pertussis* can no longer be isolated. These manifestations of pertussis set it apart from other bacterial respiratory infections and they suggest that some form of tissue sensitivity may be playing a role. Whether or not the child develops an increase in histamine sensitivity is not known. It must be kept in mind in making a comparison, that in man the histamine content of tissue is much greater than in the mouse, and that normally, sensitivity is also much greater. In the mouse, the tissue content is so low that the increased sensitivity can not be due to an additive amount of histamine released from the tissue. Whether or not the reaction is due to altered tissue susceptibility, vascular changes, a stress factor or some other factor must await further investigation. An explanation for the reaction in the mouse might help to elucidate the nature of this infection in man.

The resistance of the recovered mouse, to an intracerebral inoculation of *H. pertussis* provides further justification for the use of the latter route in testing the protective potency of pertussis vaccines in mice(6).

Summary. Mice inoculated intranasally with a culture of *H. pertussis* developed lung infections from which more than half of the mice survived. The average time of death ranged from 16 to 23 days. They developed a high degree of sensitivity to histamine comparable to that induced by pertussis vaccine, but it was slower in developing and it persisted much longer. A high percentage of the mice that died from histamine shock showed gross lung lesions and *H. pertussis* was recovered from the lungs of the majority. Most of the mice that survived this infection were resistant to an intracerebral inoculation of *H. pertussis*.

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Sulfhydryl Content of Normal Hemoglobin and Hemoglobin in in Sickle Cell Anemia.* (18683)

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Recent observations suggest that the abnormal physical and physiological properties of the erythrocytes in sickle cell anemia can be explained on the basis of abnormalities in their intracellular components. Evidence of a difference between the molecules of normal and of sickle cell hemoglobin has been obtained by electrophoresis(1). The increase in the viscosity of the whole blood of patients with sickle cell anemia which occurs on deoxygenation(2) has been reproduced in solutions of hemoglobin(3). The birefringence noted in sickled erythrocytes(4) is also a property of the tactoid aggregations which form when solutions of sickle cell hemoglobin are deoxygenated, and the tactoids themselves resemble in shape sickled erythrocytes(3).

Sickling *in vitro* of intact erythrocytes can be produced by a variety of reducing agents (5). Among these are cysteine, glutathione and BAL, which contain sulfhydryl (SH) groups(6). Sickling is inhibited by Hg^{++} and As^{+++} ions, iodoacetamide and o-chloromercuribenzoate, all of which are SH inhibitors(6,7). Thus, it appears that SH groups may be specifically concerned in the sickling phenomenon. It was of interest, therefore,

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TABLE I. Sulfhydryl (SH) Content of Normal, Sickle Cell Anemia, and Sickle Cell Trait Hemoglobins.

Hemoglobin	Normal				Sickle cell anemia					Sickle cell trait	
Patient	H.	K.	I.	Ru.W*	Ro.W.	Hz	Hr	Ho	C.	B.	S.
Moles SH/Mole hgb.	2.08	1.99	2.06	2.05	3.10	3.04	2.87	3.31	3.02	2.44	2.48
Mean SH/hgb.‡	2.04 ± .04				3.07 ± .16					2.46 ± .03	

* Normal sister of patient Ro.W.
† Mean of group given with stand. dev.

to determine whether the reduced SH content of sickle cell hemoglobin differs from that of normal hemoglobin. In the present communication are presented the results of the determination of the SH content of the hemoglobin molecules of 4 normal individuals, 5 patients with sickle cell anemia, and 2 with sickle cell trait.

Methods. The hemoglobin solutions were prepared by collecting venous blood in Alsever's solution. The cells were then washed 3 times with 0.85% sodium chloride solution, and hemolyzed with distilled water. Sodium chloride was then added to a concentration of 2.0 g % and the solutions were allowed to stand overnight in the refrigerator. The stromal precipitate was removed by centrigugation of 4°C and 16,000 RPM for 1½-2 hours. Thereafter the hemoglobin solutions were dialyzed against 0.85% saline at 4°C until they were entirely free of nonprotein reducing substances, as determined by the method of Woodward and Fry for the estimation of glutathione(8). The hemoglobin concentrations of the solutions, as determined in an Evelyn photoelectric colorimeter, were brought to about 1.5 g % with saline solution. Aliquots of 0.2 ml were titrated by the amperometric method of Kolthoff and Harris(9), modified for the determination of protein-sulfhydryl by Benesch and Benesch(10) and Weissman, Schoenbach and Armistead(11). A further modification of this method was employed in that the titrations were carried out in 0.85% saline solution. The values obtained in this medium were found to be lower and more consistent than those obtained by titration in 20% methanol, the medium used by Weissman, *et al.* All solutions were titrated at least twice with 0.001 N $AgNO_3$. In several instances the same results were obtained by titration with 0.0005 N $AgNO_3$. A molecular weight of 68,000 was used in calculating the molarity of the solutions of both normal and abnormal hemoglobins. Inasmuch as hemoglobin comprises 95% of the dry weight of the entire erythrocyte(12) the SH groups contained in other nondialyzable materials within the erythrocytes were not considered to contribute significantly to the titration values.

Results. The results of the titrations in saline of the SH content of the hemoglobin of each individual are given in Table I. Normal hemoglobin gave a mean value of 2.0 SH groups per molecule, whereas the hemoglobin of patients with sickle cell anemia gave a mean value of 3.0 SH groups per molecule. Intermediate values were obtained in the hemoglobin of the patients with sickle cell trait. In no instance did duplicate determinations vary by more than 2.0% and the mean variation of duplicate determinations in all patients with 0.82%.

When titrations were carried out in 20% methanol in saline, the values for titratable SH of the hemoglobins of all 4 groups of individuals was 4 to 5 moles per mole of hemoglobin. The values obtained from normal hemoglobin titrated in this medium did not differ significantly from those obtained from sickle cell anemia or trait hemoglobins.

In saline solutions, the SH groups of both normal and abnormal hemoglobins were

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blocked stoichiometrically with p-chloromercuribenzoate, an agent with a high degree of specificity in binding SH groups(13).

Discussion. Although a significant difference has been demonstrated between the SH titration values of normal hemoglobin and that of patients with sickle cell anemia as determined amperometrically, the data do not make clear whether this is due to a difference in the total content of SH groups or to an alteration in their reactivity.

The possibility was entertained that the 5 to 15% reticulocytes present in the blood of patients with sickle cell anemia might alter the values obtained in the determination of the SH content of the hemoglobin. This was rendered unlikely by the findings in the patients with sickle cell trait, in whom reticulocyte counts were within normal limits. In addition, hemoglobin was obtained from two patients with pernicious anemia during the period of reticulocyte response subsequent to treatment with vitamin B_{12}. These, like normal hemoglobin, contained about 2 moles SH per mole of hemoglobin.

The finding of 2 moles of SH per mole of normal human hemoglobin agrees with that reported by Hughes, who employed methylmercury iodide as a SH-blocking agent (15). The SH content of the hemoglobin molecules in the 2 patients with sickle cell trait was greater than that of normal hemoglobin but less than that of sickle cell anemia hemoglobin. This is consistent with evidence that the hemoglobin in such patients is a mixture of normal and sickle cell anemia hemoglobins(16). It was estimated that the hemoglobins from the 2 patients with sickle cell trait who were studied contained approximately 48% and 44% of abnormal hemoglobin, respectively.

The present study confirms the finding that the SH titration value of hemoglobin, as determined amperometrically in 20% methanol, is 4 to 5 moles per mole of hemoglobin (11). Denaturing procedures may uncover previously unmeasurable SH groups(17). Slight denaturation of the hemoglobin molecules, produced by 20% methanol at room temperature, may account for the higher figure that is obtained in methanol as compared with saline. The data are not adequate to explain why the difference in the sulfhydryl content of normal and sickle cell anemia hemoglobin is not demonstrable in methanol.

The capacity of p-chloromercuribenzoate to block stoichiometrically the measured SH groups of both normal and abnormal hemoglobins suggests that when the amperometric determinations are carried out in 0.85% saline solution, the method is specific for SH groups.

Conclusions. 1. The titratable SH content of hemoglobin was determined amperometrically in saline, and found to approximate 2 moles SH per mole of normal hemoglobin and 3 moles SH per mole of hemoglobin from patients with sickle cell anemia. 2. The SH content of hemoglobin from two patients with sickle cell trait was greater than that of normal hemoglobin and less than that of sickle cell anemia hemoglobin suggesting that a mixture of the two types of hemoglobin was present in these patients.

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Response of Mice to Certain Avirulent Bacteria after Exposure to Sublethal Total Body X-Irradiation.* (18684)

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It has been found previously(1,2) that whole body X-irradiation markedly lowers the resistance of mice to infection. The appearance of bacteremia following this treatment has been demonstrated by several investigators(2-6). Recently, in a careful and systematic study, Miller and associates(7) stated that during the period of greatest mortality from X-irradiation in mice "bacteria from the lower intestinal tract invaded the blood stream and produced bacteremia often of great severity." Howland and associates(8) and Miller and associates(9) have observed that some degree of protection is afforded animals against the effects of total body irradiation by means of antibiotics. Graham and Graham(10) have reported that groups of mice inoculated with 1 ml of horse serum immediately after exposure to 400 r total body irradiation evidenced greater mortality than did control animals. In the course of current investigations of the relationship of irradiation to immunity(11), it was observed that mice subjected to sublethal doses of irradiation succumbed to subcutaneous inoculation of live and heat-killed avirulent bacteria. This paper describes the initial studies dealing with this phenomenon.

Experimental procedure. Five to 6 weeks old NAMRU strain mice(12) from the animal colony of the Naval Medical Research Unit No. 1 were used in the experiments. This colony was established in 1943 and to date has been entirely free of any detectable infectious disease. The animals were irradiated by means of a constant-potential Westinghouse X-ray unit. The radiation factors were as follows: 250 KV; 15 ma; filter, 0.6 mm copper; H.V.L. 1.3 mm copper; distance from target to skin 100 cm; output in air approximately 25 r per minute. All irradiated mice were given a single dose of 350 r to the entire body. This dose was uniformly sublethal in a 30-day period. The X-ray LD_{50} for this strain of mice under the conditions employed is approximately 500 r. Control mice were

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TABLE I. Response of Normal and Irradiated Mice to Subcutaneous Inoculation with Certain Preparations.

	Mortality			
	Irradiated		Normal	
Inoculated preparations	Dead/Total	%	Dead/Total	%
I. *P. pestis*, Strain A-1122				
Administered 6 days after irradiation				
1×10^9/ml	15/15	100	7/15	47
6×10^8/ml	27/30	90	0/30	0
5.6×10^8/ml	91/100	91	17/100	17
5×10^8/ml	27/40	68	4/40	10
5×10^8/ml	37/50	74	7/50	14
1×10^4/ml*	4/15	27	0/15	0
5.1×10^1/ml†	32/50	64	5/50	10
Heat-killed‡	16/30	53	3/30	10
II. *P. pestis*, Strain K-3				
1×10^8/ml	20/30	67	1/26	4
Seitz filtrate of above culture	10/30	33	0/27	0
III. *E. coli*				
5×10^8/ml	8/15	53	0/15	0
5×10^5/ml	14/15	93	1/15	7
IV. Other substances				
Physiological saline	1/15	7	No data	No data
Heart infusion broth	0/15	0	,, ,,	,, ,,
Bovine albumin, 1%	0/15	0	,, ,,	,, ,,

* Obtained by diluting 5×10^8/ml bacterial suspension.

† Obtained by heating 5×10^8/ml bacterial suspension, but not killing all organisms.

‡ Obtained by heating 5×10^8/ml bacterial suspension, and killing all organisms.

subjected to the same manipulations as were the experimental animals except for exposure to X-rays. Avirulent *Pasteurella pestis* cultures were grown for 24 hours at room temperature in heart infusion broth (Difco) which was aerated by continuous shaking. Static cultures of *Escherichia coli* were grown in nutrient broth for 24 hours at 37°C. Heat-killed organisms were obtained by exposure of a live culture to 56°C for 2 hours. All cultures were resuspended in physiological saline after centrifugation at 2700 rpm. In the initial experiment, irradiated mice were inoculated subcutaneously with 0.2 ml of the preparations listed in Table I. Mortality over a 30-day period was used as the criterion of response. In the second experiment cultures were taken of inoculated animals as indicated below, in an attempt to elucidate the mechanism of increased susceptibility of the irradiated mice to avirulent or dead bacteria. Two series of female mice were used; the first primarily for serial sacrifice and culture, the second for mortality control animals (see Table II). In the first series, 5 mice of each of 4 groups described below, as well as one normal animal and one animal subjected only to X-irradiation were sacrificed seriatim daily for 9 days by means of nembutal, and were autopsied using aseptic technic. The 4 groups were:

(1) Sixty-five mice inoculated subcutaneously with about 6 x 10^8 live avirulent *P. pestis* strain A-1122, 6 days after irradiation.

(2) Sixty-five irradiated mice treated as in (1) except that the *P. pestis* culture used was heat-killed.

(3) 65 unirradiated mice treated as described in (1).

(4) 65 unirradiated mice treated as described in (2).

Cultures of liver, spleen, bone marrow, and heart blood (cardiac puncture) were made on horse-blood agar and in thioglycollate medium. Generic identification of *Pasteurella* cultures was made by means of pestiphage. Phage negative cultures were studid further for lactose fermentation reaction on eosin methylene blue agar.

The mortality control series was intended to indicate the 30-day lethality response of the mice to the inoculated bacteria used in series

TABLE II. Results of Cultures of Bone Marrow, Spleen, Blood, and Liver of Mice.

	Mice inoculated subcutaneously with Live *P. pestis*		Heat-killed *P. pestis*		Controls	
	Irradiated	Normal	Irradiated	Normal	Irradiated	Normal
Series 1						
No. of mice	65	65	65	65	9	9
No. died during experiment	26	3	18	3	0	0
No. cultured	39	45	40	45	9	9
Gram negative rods isolated*						
lactose +, phage —	10	4	25	0	2	0
lactose —, phage +	16	12	0	0	0	0
not tested	4	5	4	4	0	0
Gram positive cocci or rods	4	5	5	1	0	0
Negative cultures	5	19	6	40	7	0
Series 2 (Mortality controls)						
No. of mice	30	30	30	30	None	None
No. died within 30 days after inoculation	27	0	16	0	,,	,,

* When one or more organs cultured was positive for organism in question, the animal is positive for that organism.

1. Four groups of 30 mice each were treated in a manner identical to that of series 1, except that no animals were sacrificed.

Results and discussion. In the initial experiments, mortality in groups of irradiated mice inoculated with either live or heat-killed avirulent *P. pestis* and live *E. coli* was considerably greater than in groups of control animals receiving the same inoculum (Table I). Similar results were obtained by the inoculation of a Seitz filtrate of avirulent *P. pestis*. Physiological saline, nutrient broth or 1% bovine albumin did not cause death in irradiated mice.

Table II summarizes the results of the serial sacrifice experiments. No Gram-negative, lactose fermenting rods were isolated from normal mice inoculated with heat-killed cultures of avirulent *P. pestis*. However, of the 40 sacrificed irradiated mice inoculated with the same culture, 25 yielded Gram-negative, lactose fermenting rods that were not lysed by pestiphage.

Cultures of Gram-negative non-lactose-fermenting rods that were lysed by pestiphage were isolated from 12 of the 45 normal control mice,§ and from 16 of the irradiated mice, inoculated with live avirulent *P. pestis*. Four normal animals, and 10 irradiated animals, inoculated with the same culture of *P. pestis*, showed not only the presence of phage positive, Pasteurella-like organisms but also phage negative, lactose-fermenting forms. No bacteria were isolated from normal uninoculated controls, but two out of nine irradiated uninoculated mice showed the presence of Gram-negative, lactose-fermenting organisms.

Of the 65 normal mice inoculated with either live or heat-killed *P. pestis*, 3 died within the duration of the serial sacrifice experiment (9 days). During the same period, 18 of 65 irradiated mice given heat-killed *P. pestis*, and 26 of 65 irradiated animals administered live avirulent *P. pestis* succumbed to the inoculations. Since these groups served as the source of animals for daily sacrifices, the above statistics represent minimal numbers of deaths. More representative lethality data are indicated by the results of the control mortality experiment (series 2). No deaths were observed among normal mice inoculated with the bacteria, while of the mice irradiated 6 days prior to inoculation, 27 out of 30 died after injection of live *P. pestis*, and 16 out of 30 died in response to heat-killed organisms from the same culture.

Conclusions. In these experiments, death was associated with bacteremia.[||] When avirulent live bacteria were injected, the recovered organisms were either of the strain inoculated, or were apparently autogenous bacteria. However, when heat-killed bacteria were introduced, the recovered organisms were of autogenous origin only. It is known that bacteremia, identified with normal bowel inhabitants, is often an integral part of the syndrome observed in animals dying from whole body irradiation. In this instance, however, bacteremia and death were induced in animals exposed to sublethal doses of irradiation and subsequently inoculated with live avirulent, or even dead organisms generically different from the bacteria normally present in the intestines of these animals. This observation may be of importance under conditions where irradiation is a factor and vaccination is indicated.

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§ Jawetz and Meyer(13) have reported recovery of avirulent *P. pestis* from inoculated mice.

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[||] Complete identification of isolated lactose fermenting cultures was not made.

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Autoradiograms of Irregular Surfaces.* (18685)

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In studies of shell formation using Ca^{45} and P^{32} it became necessary to follow isotope distribution on the curved inner surfaces of mollusc shells. For accurate localization of radioactivity on such surfaces by means of photographic emulsions close contact between the emulsion and the surface is necessary. Photographic plates and cut film which are satisfactory for flat surfaces will not give sufficiently close contact with curved and irregular surfaces. Liquid emulsion, which might be used, has not proved suitable in our experience in providing a layer of uniform thickness; and stripping film is not sufficiently flexible to conform to irregular surfaces. We have found that emulsion sheets removed from x-ray film will serve very satisfactorily. The emulsion layer is uniform in thickness and will faithfully follow and adhere to all contours. This material is also useful for making autoradiograms of bones as well as shells. Details of the method are illustrated below with bones of the guinea pig.

Eastman Industrial X-ray Film, Type A, in strips 1⅞ x 16 inches was used. This film is more sensitive than Type M and less sensitive than No-Screen and Type K. Because of its smaller grain size the resolution is better than the latter two(*1*,*2*). All operations are carried out in a darkroom using a Wratten safelight, Series OA. After cutting the film into pieces of appropriate size they are placed in distilled water at 30°C for about 5 minutes to soften the emulsion. By means of a piece of plastic or glass with a polished edge the emulsion can now be completely removed in a sheet by beginning at one end of the film strip and pushing the emulsion ahead of the instrument. With a satisfactory edge the sheet of emulsion is removed without defect and with none remaining on the backing. The emulsion is then placed in distilled water at 20°C where it will toughen and wrinkles will disappear. The emulsion is applied by floating it onto the surface to be studied. As the object is lifted from the water the sheet of emulsion will become closely applied to all contours (Fig. 1 and 2). Any wrinkles can be smoothed with a camel's hair brush. Leaching of radioactive material from shell and bone during the exposure to distilled water and to the wet emulsion will presumably be minimal.

After the emulsion is dry the preparations are stored in light-tight boxes for a suitable

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* This work was supported by a grant from the Atomic Energy Commission.

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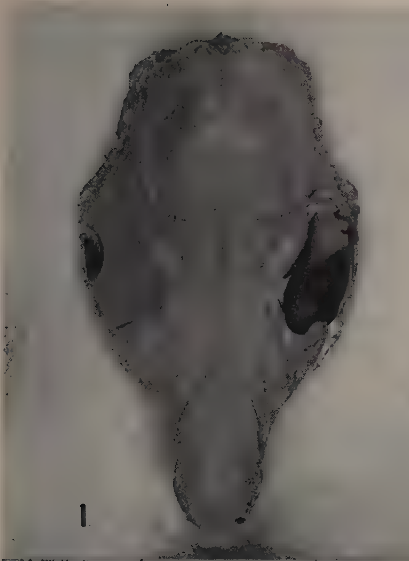
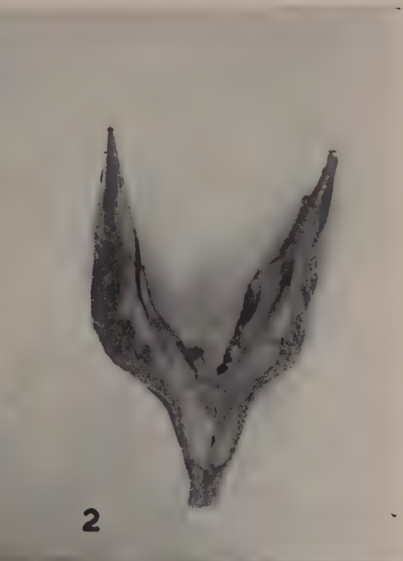


1



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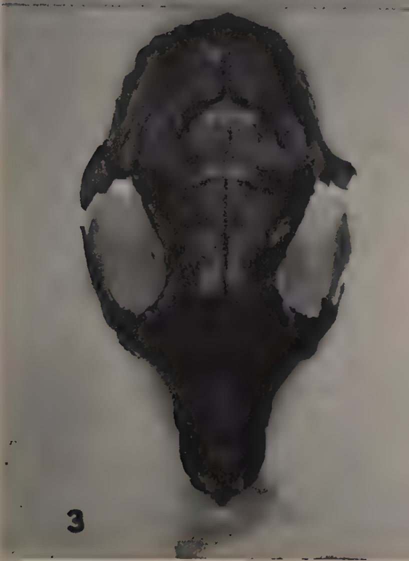

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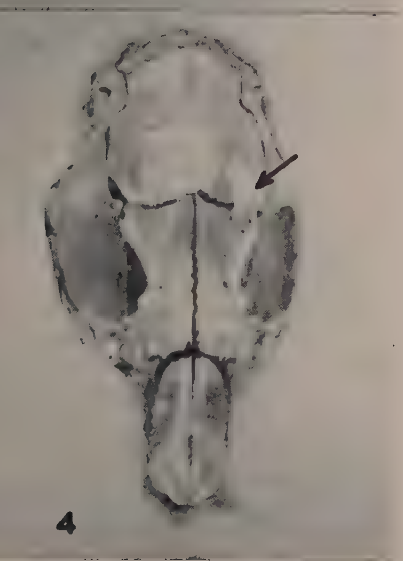

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FIG. 1 and 2. Guinea pig skull and lower jaw covered with sheet emulsion from Eastman Industrial X-ray Film, Type A. Emulsion left uncleared to show its conformity to contours.

FIG. 3. Autoradiogram of guinea pig skull showing non-uniform distribution of P^{32}. A similar distribution of P^{32} has been found in 8 skulls. Eastman Industrial X-ray Film, Type A. Highlights interfere somewhat with the accurate representation of darkening in this photograph. 100 μc P^{32} injected intraperitoneally; animals sacrificed after 20 hr. Skulls heated very briefly in boiling water for cleaning. Emulsion exposed 24 hr.

FIG. 4. Non-radioactive guinea pig skull covered with emulsion, developed and cleared. Black lines at sutures are the result of chemical or physical action. Arrow indicates an area including a suture from which the emulsion has been removed.

exposure time. The emulsion is then developed in D-19 developer(2) while still on the specimen. Fixation is carried out in the following alkaline solution: sodium thiosulphite, 960 g; sodium sulphite, desiccated, 50 g; water, 4 1. Since the solutions are alkaline except for a very brief acid stop-bath, no decomposition of shell or bone will occur. Development of the autogram while on the specimen obviously facilitates the accurate localization of radioactive areas (Fig. 3). However, qualitative visualization of regional differences in radioactivity of a specimen is, of course, limited to uniformly light colored material. Quantitative data may be obtained by counting developed silver grains while the emulsion is still on the specimen or by removing it to a glass plate and making densitometer measurements. For the latter procedure reference marks may be made on the emulsion and the underlying material with a pointed instrument and the emulsion sheet removed immediately after development while still moist. This is easily accomplished by loosening at the edges. Radioactive ink (3) applied to the shell or bone will also provide reference points on the isolated emulsion.

Counting of silver grains in the emulsion on radioactive oyster shells may be facilitated by making photomicrographs of the intact preparation using a 20 x objective and a 15 x ocular and a Whipple disc(4). The negatives are then projected with a photographic enlarger. Quantitative measurements of Ca^{45} and P^{32} in oyster shells have been made in this way. For surfaces with more extreme contours the emulsion may be removed for photographing.

During removal from the backing the emulsion increases in linear dimensions by about 25%, a property shown also by stripping plate emulsion(1). We have studied this by ruling small squares on the emulsion prior to removal and making measurements after consecutive steps in the procedure. Such measurements show that the stretching which occurs after removal of the emulsion from the backing is uniform in all directions. No apparent stretching or distortion occurs as a result of applying the film to a curved surface, subjecting it to development procedures, or by removing the developed emulsion to a glass plate. However, localized stretching of the emulsion probably occurs in conforming to marked curvatures.

Because of direct contact between the emulsion and the specimen the possibility of darkening through physical or chemical action must be considered and suitable control exposures made on non-radioactive material. Non-radioactive oyster shells coated with emulsion and run through the entire procedure exhibited no darkening of the emulsion. However, in a series of 14 non-radioactive guinea pig skulls 11 showed varying degrees of darkening at sutures. The most extreme case is illustrated in Fig. 4. Darkening also occurred over certain areas of the lower jaw. Further investigation of the cause of this darkening would be interesting but is beyond the scope of this study. The elimination of this effect presumably could be approached by the application of film lacquer or some other inert substance to the surface to be autographed. However, this would reduce soft beta radiation from the surface; and if the thickness of the applied material were not uniform, the autogram would not give a true record of the radioactivity.

Summary. 1. Autoradiograms of irregular surfaces of mollusc shells and curved surfaces of bone can be made without difficulty by applying sheets of emulsion removed from Eastman Industrial X-ray Film, Type A. The emulsion is developed while on the object, facilitating the localization of radioactive areas, and may be removed for densitometric measurements if desired. 2. Darkening of the emulsion may occur as a result of direct contact with sutures and certain definite regions of non-radioactive bone. Accordingly, in making autoradiograms by this method control exposures on non-radioactive bone are required to evaluate this effect.

We wish to thank Dr. George A. Boyd of the Oak Ridge Institute of Nuclear Studies for his helpful criticisms of the manuscript and the method.

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Renal Clearance of Endogenous Phosphate in Infants and Children. (18686)

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While studying patients with altered phosphate metabolism, it became necessary to establish values for renal clearance of phosphate in normal pediatric subjects. Although values were available for adults from the studies of Ollayos and Winkler(1) and Blatherwick and collaborators(2), no published data were available for infants and children. Since the initiation of our study one report(3) has presented data which compared clearance of endogenous phosphate in a group of 9 newborn infants and one 5-month-old infant with adult clearances. Since no studies of the glomerular filtration rate were performed simultaneously, the relationship of glomerular filtration to tubular reabsorption was not established. Gardner and coworkers(4), while studying tetany in the newborn, performed renal phosphorus clearances in one full term infant and in one premature infant and found that the newborn infant's kidneys respond to parathyroid hormone injections by increased urinary excretion of phosphorus. McCrory *et al.*(5) indicated that the elevation of endogenous serum phosphorus in newborns could be related to the reduced glomerular filtration rate. The injection of parathyroid preparations caused an increase in renal excretion of phosphorus and an associated decrease in reabsorption.

Methods. Our subjects were patients from the pediatric service of the University of Illinois Research and Educational Hospital. The newborn infants were normal; the older infants and children were hospitalized for the surgical treatment of cleft palate, strabismus, or for tonsillectomy. In all instances other abnormalities were excluded insofar as possible by physical examination, urinalysis, blood chemistry and complete blood counts. The infants were fed evaporated milk formulae and the children received a general ward diet. All subjects were fasted for a period of at least 3 hours prior to the clearances. No feedings were permitted during the period of study. Urine specimens were obtained by catheterization. Air was injected via the catheter into the bladder to facilitate the collection of residual urine. Blood specimens were obtained at the midpoint of the 20-minute urine collection periods, except for the infants under one year, for whom one hour periods were employed. The femoral and jugular veins were the sites chosen for venipuncture in infants; the antecubital vein was utilized in older children. Blood and urine were analyzed for inorganic phosphorus by the method of Fiske and SubbaRow(6), and inulin was determined on both urine and blood by the method of Hubbard and Loomis(7). The doses of inulin employed for children and adolescents were proportionate to those suggested by Goldring and Chasis(8) for adults. These doses were calculated on the basis of body weight with the assumption that 30 ml of a 10% solution of inulin is the priming dose and that 70 ml of a 10% solution is the sustaining dose for a 150-lb. adult. The volume of 0.9% saline in the priming solution employed in the older group was 250 ml flowing

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TABLE I. Inulin and Phosphate Clearances in Infants Up to 5 Days of Age.

Subjects	Age in days	Area, sq.m.	Serum P, mg %	Urine P, mg %	Urine vol., ml/min.	Clear P, ml/min.	Inulin clear, ml/1.73 sq.m./min.	% of P resorbed	% of P excreted
P.	3	.21	11.9	15.8	.14	2.37	—	—	—
B.	3	.19	8.1	11.5	.21	4.50	28.2	90.4	9.06
B.B.	4	.19	7.6	35.0	.13	5.4	—	—	—
			7.6	25.0	.14	4.4			
M.	4	.19	7.8	23.2		8.4	58.5	86.0	14.0
			7.8	24.2	.24	6.7	54.0	87.6	12.4
A.	4	.20	7.8	46.2	.22	11.0	32.5	66.2	33.8
B.L.	4	.21	7.6	30.0	.15	3.2	—	—	—
			7.6	40.0	.27	4.6			
B.A.	5	.24	7.8	28.7	.20	5.4	—	—	—
			8.2	35.0	.10	3.1			

TABLE II. Inulin and Phosphate Clearances in Subjects 21 Days to 20 Months of Age.

Subjects	Age	Area, sq.m.	Serum P, mg %	Urine P, mg %	Urine vol., ml/min.	Clear P, ml/min.	Inulin clear, ml/1.73 sq.m./min.	% of P resorbed	% of P excreted
G.	21 days	.22	7.8	81.0	.17	13.1	—	—	—
G.	24 ”	.23	7.6	57.0	.21	11.7	—	—	—
			7.6	80.0	.18	14.2	60.0	76.4	23.6
B.	5 wk	.23	5.5	49.1	.36	24.0	69.0	65.3	34.7
			5.4	35.6	.66	32.2	76.0	57.7	42.3
			5.8	16.9	.16	34.5	82.5	57.0	43.0
D.	3 mo.	.30	4.9	27.8	1.03	31.9	141.0	77.4	22.6
			4.7	32.2	.76	29.6	108.0	72.6	27.4
			4.7	56.3	.43	29.6	127.0	76.7	23.3
C.	6 ”	.36	7.9	128	.16	13.6	—	—	—
J.	7 ”	.38	8.3	245	.11	14.4	—	—	—
S.	20 ”	.39	5.5	140	1.3	15.4	137	88.8	11.2
			5.4	490	.26	10.4	94	89.0	11.0

at a rate of 10 ml per minute. This was followed by a sustaining dose of 500 ml at a rate of 4 ml per minute. For infants we employed doses of inulin varying from 2 to 5 ml of a 10% solution for the priming dose, depending upon body weight. This dose was diluted to 10 ml in 0.9% saline prior to injection. Depending upon body weight of the subject, the sustaining solution contained from 4 to 10 ml of a 10% solution of inulin, which was dissolved in 150 to 200 ml of 0.9% saline and was allowed to run at a rate of 40 to 60 ml per hour. In the experiments in which inulin was not administered, water was given orally to promote a diuresis. No glucose containing solutions were employed for any of the subjects.

Results. Our studies indicate that the renal clearances of inorganic endogenous phosphate in the newborn period are relatively low. The clearances in 7 infants from 3 to 5 days of age averaged 5.37 ml/min. per 1.73 square meters of body surface (Table I). In order to determine the age at which phosphate clearances approximate adult values, a group of older infants ranging in age from 21 days to 20 months was studied. These clearances averaged 21.1 ml/min. per 1.73 sq. m. of body surface, thus being comparable to values observed in older children and adults (Table II). Nineteen children ranging in age from 4 to 13 years of age were studied. Six of these subjects in whom simultaneous inulin clearances were performed had an average phosphate clearance of 14.67 ml/min. per 1.73 sq. m. of body surface (Table III). Thirteen additional subjects in this age group had an average clearance value of 13.0 ml. (Table IV).

The tubular reabsorption of phosphate was

TABLE III. Inulin and Phosphate Clearances in Children.

Subjects	Age, yr	Area, sq.m.	Serum P, mg %	Urine P, mg %	Urine vol., ml/min.	Clear P, ml/1.73 m./min.	Clear inulin, ml/1.73 m./min.	% of P resorbed	% of P excreted
T.	10	1.16	5.8	4.45	17.5	19.9	138.0	86.0	14.0
			5.4	5.83	11.0	17.4	140.6	87.0	13.0
F.	12	1.24	4.9	6.25	4.25	22.7	133.8	83.0	17.0
			5.0	5.25	12.5	20.7	125.8	83.6	16.4
			4.5	11.85	14.0	20.5	107	83.3	16.7
C.	12	1.32	4.6	76.0	.95	20.56	112	81.8	18.2
			4.5	13.0	5.25	17.78	102.3	82.7	17.3
			4.5	8.0	5.23	12.1	94.0	87.2	12.8
C.W.	9	1.15	4.9	9.5	4.6	13.3	135.6	90.3	9.7
			4.8	3.2	13.0	12.9	130.5	90.2	9.8
			4.8	5.9	5.5	10.2	104.5	90.3	9.7
S.	12	1.67	5.1	6.4	6.5	8.6	95	91.0	9.0
			5.2	7.4	6.75	9.9	130	92.4	7.6
			5.1	6.0	6.25	7.6	132	94.2	5.8
			5.2	6.0	6.25	7.6	136	94.4	5.6
I.	9	.98	5.8	41	1.1	13.5	92	85.5	14.5
			5.0	28.7	1.2	12.1	86.8	86.1	13.9
			5.4	11.0	4.7	16.7	86	80.6	19.4

calculated by comparing the inulin clearances with the phosphate clearances. Thus of the six children and adolescents in whom both clearances were simultaneously determined an average of 87.7% was reabsorbed by the renal tubules. In subjects from 21 days to 20 months of age, an average of 74.7% was reabsorbed. In 3 infants under 5 days of age an average of 82.6% of the phosphate was reabsorbed by the tubules.

Discussion. Although Schloss and Crawford(9) demonstrated some years ago that the newborn infant has a relatively low urinary phosphate output, it was not until the recent publication of Dean and McCance(3) that endogenous phosphate clearance data for pediatric patients became available. The values reported by these authors are in agreement with those described in this study. The data presented indicate that renal clearance of phosphate in newborn infants is relatively low and thus is in keeping with renal functional immaturity characteristic for this age period(10). This may account, in part, for the higher serum phosphate levels of young infants. The 4 infants from 3 to 12 weeks of age had clearances comparable to adult values. Since Rubin(11) and West(12) and their respective groups have indicated that renal function, as measured by inulin and diodrast clearances, does not attain maturity until 10 weeks to 2 years of age, it would seem that phosphate excretion tends to attain mature values earlier than most renal functions. The work of Gardner *et al.*(4) showed that a full term infant had varying phosphorus clearances depending on the kind of milk consumed. During the days when human milk was ingested, a recalculation of their data expressed in ml/1.73 sq. m./min. shows that the phosphorus clearances were 0.53 ml and 0.395 ml on the 7th and 8th day of life, respectively. On a commercial cow's milk product, the clearances were 1.3, 1.7, and 8.1 ml before the first 11 days of life. These figures are reasonably within range of the values in this study. Clearances during the third week of life when the infants were on human milk were 1.5, 0.75, and 0.25 ml. Gardner and coworkers explain the differences in phosphorus clearances between breast milk and the cow's milk product because of compensatory hyperplasia of parathyroid tissue associated with cow's milk feedings. McCrory *et al.*(5) found that there is no evidence available to completely

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TABLE IV. Phosphate Clearances in Children.

Subjects	Age, yr	Area, sq.m	Blood P, mg %	Urine P, mg %	Urine vol., ml/min.	Clear P, ml/1.73 m. per min.
N	4	.65	6.0	40.0	8.0	17.7
			6.0	53.7	2.8	23.8
			6.0	11.0	5.0	23.9
D.	5	.75	5.7	55.0	.72	15.6
			6.0	30.0	1.9	18.1
R.	6	.70	5.8	21.0	3.71	33.0
			6.0	16.0	5.5	36.0
M.	9	1.09	5.2	10.7	2.39	8.7
			5.7	12.7	3.44	12.0
			6.2	22.4	2.05	11.7
B.	9	.90	6.4	33.0	1.86	18.7
			6.3	35.0	1.5	16.1
J.	9	1.13	5.75	80	.52	11.0
			5.75	100	.48	12.7
			5.55	18.5	2.45	12.4
C.	9	1.17	5.1	14.9	4.3	18.6
			5.2	31.8	3.0	26.9
C.S.	10	1.07	3.8	45	4.2	8.3
			3.9	38	.7	11.1
			4.1	17.5	1.6	11.5
C.E.	10	.96	5.8	10.0	3.1	8.6
			6.1	60.0	.47	8.2
			6.3	11.0	3.1	10.1
B.L.	10	1.08	7.8	32.0	2.7	17.6
			7.4	11.0	9.2	21.2
			7.4	7.5	9.7	15.5
			7.2	7.5	10.4	17.2
S.	12	1.67	5.5	13.9	3.4	8.8
			5.4	9.5	5.0	7.0
R.B.	13	1.11	4.1	6.5	3.3	8.2
			4.2	13.0	4.0	19.2
			4.7	19.0	3.3	20.7
O.	13	1.07	5.9	6	2.3	15.36
			5.9	7.0	6.0	11.7
			6.1	18.5	9.0	11.2

substantiate the hypothesis that infants with endogenously elevated serum phosphorus levels or with tetany are different from normal infants in their parathyroid function if variations in tubular reabsorption of phosphorus is an accurate reflection of such function.

The values for phosphate clearance in children and adolescents corresponded closely to clearances determined in adults by other investigators. Ollayos and Winkler(1) found an average clearance of 15 ml/min. per 1.73 sq. m. in adults. Blatherwick, Bell, and Hill(2) observed the renal clearance of endogenous phosphate to range from 5.8 to 13.3 ml/min. in normal adult subjects. Dean and McCance(3) found values from 8 to 38 ml/min. per 1.73 sq. m. in normal adults. Thus from all reported studies, phosphate clearance shows a considerable range of variation in normal individuals. It has been demonstrated that this range of variation in the clearance of phosphate is not related to blood level unless it has been elevated by a phosphate infusion(1).

Gamble(13) has determined that the tubular reabsorption of phosphate averages 83% of the glomerular filtrate in normal adult subjects. In our study the tubular reabsorption was observed to be 87.7% of the glomerular filtrate in children and adolescents. The older infants studied had an average tubular reabsorption of phosphate of 74.7% of the glomerular filtrate. This would seem to reflect some immaturity of renal tubular function. The newborn infants had a higher value of 82.6%

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tubular reabsorption. This higher value in newborn infants may be more apparent than real, since the tubules presumably have presented to them a relatively smaller load in the glomerular filtrate. Since clearance periods in this age period are admittedly inadequate, no definite conclusions on this point can be drawn.

Summary. 1. The clearance of endogenous inorganic phosphate is relatively low in the newborn period. 2. The clearances of phosphate in older infants, children and adolescents approximate those of the adult. 3. The tubular reabsorption of phosphate was found to average 87.7% of the glomerular filtrate in children and adolescents. 4. The clearances of phosphate at all ages have a wide variation.

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Fate and Excretion of Adrenocorticotrophic Hormone.*† (18687)

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The fate and excretion of adrenocorticotrophic hormone (ACTH) have assumed practical as well as academic significance with the extensive use of the hormone in the treatment of disease. Porcine ACTH disappears rapidly from the blood of man following its intravenous injection(1). Two hours after its administration, only 2% of the biological activity of the injected hormone remains in the vascular compartment; after 3 hours, ACTH activity is no longer detectable in the blood. The hormone is not excreted in appreciable quantities in the urine. Essentially the same results have been obtained in the rat following the administration of porcine ACTH(2) and sheep ACTH(3).

It is not possible to decide from the results of the above experiments whether the rapid disappearance of ACTH activity from the blood stream is a characteristic of the endogenously secreted hormone or is due to the fact that in each instance a protein, foreign to the recipient species, was injected. In order to exclude the latter possibility the present experiments were designed to determine the fate and excretion of rat ACTH injected intravenously into the rat.

Methods. Rat ACTH was prepared as follows. Adult male rats from the Sprague-Dawley farm, weighing 250 to 400 g, were decapitated and the pituitaries carefully removed from the sella turcica. The anterior lobes were dissected away from the posterior lobes and ground in a 0.9% sodium chloride solution made acid to 0.1 M with hydrochloric acid. The mixture was centrifuged and the supernatant solution of ACTH placed in a boiling water bath for one hour. The final concentration of the solution of rat ACTH was 2 anterior pituitaries per ml. Each 0.5 ml of extract had the biological activity equivalent to approximately 100 μg of the ACTH standard, La-1-A. All potencies and doses are expressed as μg equivalents of La-1-A. The confidence limits of the bioassays to be presented below are based on a p value of 0.67. The male rats employed for the study of the distribution of injected ACTH were obtained from the Sprague-Daw-

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† The authors are indebted to Dr. Louis S. Goodman for his many helpful comments and criticisms.

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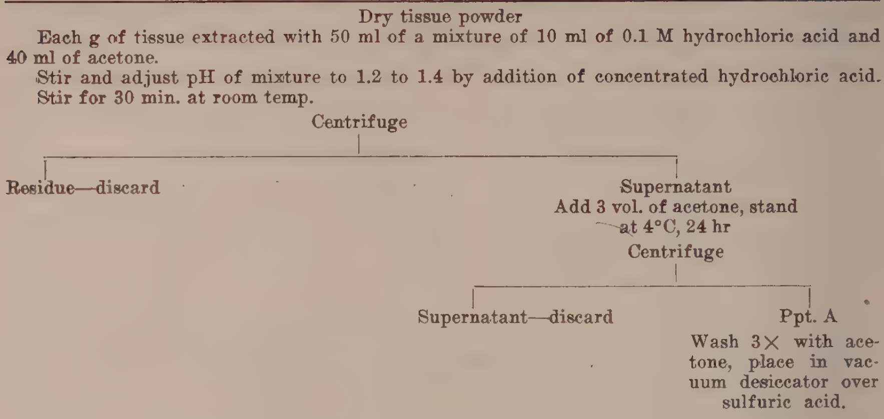
FIG. 1. Extraction and Precipitation of ACTH Activity from Tissue.


Dry tissue powder
Each g of tissue extracted with 50 ml of a mixture of 10 ml of 0.1 M hydrochloric acid and 40 ml of acetone.
Stir and adjust pH of mixture to 1.2 to 1.4 by addition of concentrated hydrochloric acid.
Stir for 30 min. at room temp.
Centrifuge
Residue—discard
Supernatant
Add 3 vol. of acetone, stand at 4°C, 24 hr
Centrifuge
Supernatant—discard
Ppt. A
Wash 3× with acetone, place in vacuum desiccator over sulfuric acid.

ley farm and weighed 250 to 350 g. The animals were lightly anesthetized with ether, injected intravenously (saphenous vein) with a solution of rat ACTH (0.5 ml per 100 g body weight) and allowed to recover from anesthesia. At 5 or 15 minutes following injection of hormone the animals were again lightly anesthetized with ether and blood was withdrawn from the abdominal aorta into a heparinized syringe. The animals were then sacrificed and the liver, kidneys and adrenals prepared for analysis. About 0.25 ml of 0.1 M hydrochloric acid was injected into the bladder and the contents of the organ were withdrawn and stored in a refrigerator until bioassayed. The tissues were frozen in dry ice immediately after removal from the animal, lyophilized and ground to a fine powder. Aliquots of the powder were extracted for ACTH according to the flow sheet presented in Chart 1. Precipitate A, the ACTH concentrate, was dissolved in 0.9% sodium chloride solution made acid to 0.1 M with hydrochloric acid and injected into the test animals for the determination of ACTH potency. Blood, urine and tissue concentrates were assayed for adrenocorticotrophic activity by the adrenal ascorbic acid-depletion test in hypophysectomized rats(4). The experiments to be reported below are designated by numbers which represent the dates on which they were performed. This is done advisably because each assay requires its own reference standard; in quantitative work it is not permissible to compare the individual adrenal ascorbic acid-depletion figures of an assay done on one day with those obtained on a different day.

Results. Blood. The following experiments were conducted in order to determine the stability of rat ACTH in rat blood *in vitro*. Heparinized blood was collected from several untreated rats and pooled. When injected into test assay animals in a dose of 3.0 ml per 100 g body weight, this blood induced no decrease in adrenal ascorbic acid. Rat ACTH was dissolved in the pooled blood to a final concentration of 1.0 μg per 0.5 ml or 4.0 μg per 0.5 ml. The 2 samples were incubated at 37°C for 1 hour. At the end of incubation they were cooled to 4°C and maintained at this temperature until bioassayed. Two dose levels, 1.0 and 4.0 μg, were employed in the assay, *i.e.*, each test rat was injected with 0.5 ml of the appropriate blood sample per 100 g body weight. The reference standard employed was rat ACTH incubated in phosphate buffer, pH 7.5, at 37°C for one hour at concentrations of 1.0 and 4.0 μg per 0.5 ml. The results are presented in Table I. Exp. 4-25-50 indicates that rat ACTH is relatively stable in phosphate buffer. Rat ACTH incubated in rat blood was estimated to be 1.10 times as potent as the hormone

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4. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.

TABLE I. Stability of Rat ACTH in Rat Blood *in Vitro*.

Exp. 1950	Preparation	Dose µg	Adrenal ascorbic acid depletion mg per 100 g tissue*	Estimate of potency with confidence limits % of specific control
4-25	Rat ACTH unincubated acid extract	2 .5	113, 113, 120 57, 98, 37, 56, 75	—
	Rat ACTH incubated in phosphate buffer, pH 7.5, 4 hr at 37°C	2 .5	113, 81, 122, 76, 104 52, 35, 56, 68	77 (60 to 101)
5-2	Rat ACTH incubated in phosphate buffer, 1 hr at 37°C	4 1	101, 131, 116, 79, 99 57, 58, 49, 58, 65	—
	Rat ACTH incubated in rat blood, 1 hr at 37°C	4 1	113, 156, 136, 145, 108, 104 17, 39, 53, 79, 21, 55	110 (93 to 131)

* Each figure in this column represents the difference between the concentration of ascorbic acid in the left adrenal, which is removed immediately prior to injection of the test material in the assay rat, and the right adrenal which is removed one hour after injection of the test substance.

TABLE II. Adrenocorticotrophic Activity of Blood from Rats Injected with Rat ACTH.

Exp. 1950	Preparation	Dose	Adrenal ascorbic acid depletion mg per 100 g tissue*†	Estimate of potency in relation to specific control,—with confidence limits
5-9 Intact rats	Rat ACTH in phosphate buffer	4 µg 1 µg	132, 111, 126, 114, 117, 114 36, 23, 51, 52	—
	Blood 5 min. after ACTH inj.	3 ml .75 ml	81, 120, 161, 95, 101, 121 61, 39, 29, 77, 43	1 ml of blood equivalent to 1.23 (1.09 to 1.39) µg
	Blood 15 min. after ACTH inj.	3 ml	5, −6, 26, 11	1 ml of blood equivalent to less than 0.10 µg
6-13 Intact rats	Rat ACTH in phosphate buffer	4 µg 1 µg	96, 160, 131, 176, 188, 135, 137 78, 90, 79, 75, 59	—
	Blood 5 min. after ACTH inj.	3 ml .75 ml	109, 127, 94, 161, 135 68, 20, 21, 67, 46, 39	1 ml of blood equivalent to .81 (.66 to 1.00) µg
6-15 Adrenalectomized rats	Rat ACTH in phosphate buffer	4 µg 1 µg	149, 149, 107, 113, 119 111, 41, 94, 76, 100, 69	—
	Blood 15 min. after ACTH inj.	3 ml	12, −3, −14, 6	1 ml of blood equivalent to less than .10 µg

* See footnote Table I.

† Negative values indicate a greater conc. of ascorbic acid in the right than in the left adrenal.

incubated in phosphate buffer (Exp. 5-2-50). The confidence limits (0.93 to 1.31) are such that this increase is not significant and it may be concluded that rat ACTH is stable *in vitro* in rat blood for at least 1 hour at body temperature. Recipient rats were injected intravenously with 0.5 ml of rat pituitary extract (equipotent with 100 µg of standard), per 100 g of body weight. The duration of injection was 15 seconds. Blood was withdrawn 5 or 15 minutes after ACTH administration. The blood of 6 rats, the number used in each group, was pooled and injected into hypophysectomized assay animals. The results are presented in Table II. After 5 minutes the concentrations of ACTH in the blood were equal to 1.23 (Exp. 5-9-50) and 0.81 (Exp. 6-13-50) μg per ml. These values are in satisfactory agreement with each other when one considers that ACTH disappears rapidly from the blood and that the interval between ACTH injection and blood withdrawal may have varied by plus or minus one minute.

If it is assumed that injected ACTH is not excreted or inactivated, and that it does not enter cells, the concentration of the hormone in the extracellular fluid should equal 5 μg per ml (100 μg, the amount injected per 100 g of rat, divided by 20 ml, the estimated extracellular fluid volume per 100 g of rat). Five minutes after administration of the hormone, the actual concentration of ACTH in whole blood was equal to 1.0 μg per ml (average of 1.23 and 0.81 μg per ml). If one assumes a hematocrit of 50% and zero concentration of ACTH in erythrocytes, the concentration in plasma may be estimated to be about 2.0 μg per ml; this means that only 40% of the injected hormone remained in the extracellular fluid compartment. Obviously, the assumption that the hormone is not excreted, inactivated or taken into cells is not permissible. Fifteen minutes after ACTH was injected, biological activity could not be detected in the blood. In terms of the sensitivity of the assay method each ml of blood had less than 0.10 μg of ACTH.

The adrenal cortex is the only tissue known to respond to ACTH. Inasmuch as some investigators assume that the site of action of a hormone may also be the locus of degradation, it was decided to ascertain whether the presence of the adrenals is concerned with the rapid disappearance of the hormone from the blood. The results are presented in Table II. Fifteen minutes after the administration of ACTH to adrenalectomized rats, biological activity was not detectable in the blood. Thus the adrenal glands do not appear to be responsible for the rapid disappearance of ACTH from the circulation, but the results do not rule out the possibility that injected ACTH has a slightly longer sojourn in the blood of adrenalectomized rats.

Tissues. The results are presented in Table III. The kidney of uninjected rats contains a substance which depletes the adrenal ascorbic acid content of the hypophysectomized rat (Exp. 9-5-50 and 9-7-50). A very approximate estimate indicates that concentrate (Ppt. A, Chart 1) obtained from 0.2 g of dried kidney tissue elicited a response equal to that of 0.5 μg of standard. A dose of 0.05 g of kidney tissue evoked a negative response (Exp. 9-7-50). No quantitative assay has been performed to determine the exact potency of kidney tissue from normal uninjected rats. Furthermore, it is not possible to state with certainty the nature or the origin of the kidney substance. The animals were decapitated and there was, therefore, no opportunity for stress to induce an acceleration of ACTH discharge from the pituitary. If the substance is of pituitary origin, it must represent an accumulation in the kidney prior to sacrifice. Liver tissue from uninjected rats in a dose of 0.2 g did not deplete adrenal ascorbic acid (Exp. 9-5-50).

It was of some importance to determine the percentage recovery to be expected with the method presented in Chart 1 for extracting and precipitating ACTH activity from rat tissues. Rat ACTH was added to liver tissue obtained from normal uninjected rats. The mixture was processed according to the scheme presented in Fig. 1. From the data of Exp. 9-7-50 it appears that the method is capable of recovering most if not all of the ACTH activity. The recovery of added ACTH was 146%. Unfortunately, the assay was relatively inaccurate (confidence limits 67 to 321%). However, even the low figure of 67% represents an acceptable recovery of added hormone. Kidney removed 5 or 15 minutes after injection of ACTH was remarkably potent in biological activity. Such tissue in a dose of 0.1 g evoked a response greater than that to 2 μg of standard (Exp. 9-13-50). Even 0.025 g of kidney tissue elicited a marked response (Exp. 9-20-50). In Exp. 10-5-50, 0.02 and 0.005 g dose levels caused responses of a magnitude suitable for statistical analysis. Each g of the dry 5-minute kidney tissue was equal in potency to 112 (86 to 147) μg of ACTH and each g of the dry 15-minute kidney tissue was equal

TABLE III. Adrenocorticotrophic Activity of Tissues from Rats Injected with Rat ACTH.

Exp. 1950	Preparation	Dose	Adrenal ascorbic acid depletion mg per 100 g tissue*	Estimate of potency in relation to specific control
9-5	Rat ACTH	4 μg	116, 155, 118, 125	—
	Kidney from uninj. rats	.2 g†	66, 89, 89, 95	.2 g = .5 μg (very approx.)
	Liver from uninj. rats	.2 g	–12, –15, –1, –2, 8, –18	.2 g<.25 μg
9-7	RAT ACTH	2 μg .5 μg	107, 148, 132, 118, 127, 108 101, 121, 93, 106, 111, 121	—
	ACTH + liver from uninj. rats	4 μg 1 μg	116, 193, 209, 103, 151 122, 137, 85, 120, 119	146 (67 to 321) % recovery
	Kidney from uninj. rats	.2 g .05 g	44, 70 1, 14, –28, 10	.2 g = .25 μg (very approx.)
9-13	Rat ACTH	2 μg .5 μg	141, 131, 107, 117, 109 26, 49, 10, 29, 70	—
	Kidney 5 min. after inj. of ACTH	.1 g	157, 162, 196, 162, 143, 153	.1 g>2 μg
	Kidney 15 min. after inj. of ACTH	.1 g	97, 157, 122, 126, 144, 159	.1 g>2 μg
	Liver 15 min. after inj. of ACTH	.2 g	–3, 13, 4, 5	.2 g<.25 μg
9-15	Rat ACTH	2 μg .5 μg	132, 152, 108, 160, 128 88, 32, 68, 41, 101	—
	Liver 5 min. after inj. of ACTH	.2 g	52, –20, –6, –1, 0	.2 g<.25 μg
	Adrenal 5 min. after inj. of ACTH	.035 g	138, 98, 121	.035 g = 2 μg (very approx.)
	Adrenal 15 min. after inj. of ACTH	.033 g	34, 81	.033 g = .5 μg (very approx.)
9-20	Rat ACTH	2 μg .5 μg	139, 90, 107, 160, 122, 102 83, 100, 81, 48, 62, 96	—
	Kidney 5 min. after inj. of ACTH	.1 g .025 g	147, 153, 161, 136, 178, 154 173, 132, 204, 193, 92, 192, 124	.025 g>2 μg
	Kidney 15 min. after inj. of ACTH	.1 g .025 g	149, 124, 199, 144, 147 158, 107, 100, 141, 167, 151	.025 g = or>2 μg
9-22	Rat ACTH	2 μg .5 μg	133, 137, 105, 97, 133 71, 99, 82, 43, 40	—
	Liver 5 min. after inj. of ACTH	.2 g	0, 51, –23, 4	.2 g<0.25 μg
	Liver 15 min. after inj. of ACTH	.2 g	0, 10, 30, –32	.2 g<0.25 μg
	Adrenal from uninj. rats	.033 g	–12, 0, 43	.033 g<0.25 μg
10-5	Rat ACTH	2 μg .5 μg	183, 177, 135, 132, 100 112, 70, 60, 92, 32, 42	—
	Kidney 5 min. after inj. of ACTH	.02 g .005 g	210, 148, 140, 116, 110 108, 67, 78, 68, 77, 71	1 g kidney tissue = 112 (86 to 147) μg
	Kidney 15 min. after inj. of ACTH	.02 g .005 g	172, 151, 114, 120, 140 102, 41, 62, 60, 42	1 g kidney tissue = 89 (71 to 111) μg

* See footnote Table I.

† Dry wt tissue from which concentrate was obtained.

TABLE IV. Distribution of Injected Rat ACTH Throughout Tissues and Body Fluids of Rat.

Amt. ACTH inj., μg	Time after ACTH inj., min.	Total quantity of ACTH μg in: Extracellular fluid	Kidneys	Liver	Adrenals	Urine
100	5	40*	20	<1	.2	0
100	15	<4*	15	<1	.05	0

Calculated per 100 g rat; extracellular fluid 20 ml; dry wt of kidneys, 0.167 g; dry wt of liver, 1.0 g; dry wt of adrenals, 0.0033 g.

* Based on assumption that there is no ACTH in erythrocytes (see text).

in potency to 89 (71 to 111) μg of ACTH.

In contrast to kidney tissue, the liver contained no appreciable ACTH activity at 5 or 15 minutes after ACTH injection (Exp. 9-13-50, 9-15-50, 9-22-50). A dose of 0.033 g of adrenal from normal uninjected rats did not produce a decrease in adrenal ascorbic acid (Exp. 9-22-50). On the other hand, the adrenals of rats injected with ACTH contained appreciable ACTH activity. The limited amount of tissue available has made it impossible to obtain an accurate estimate of the ACTH activity of this tissue. The assay of single doses of adrenal gland (0.035 g from the 5-minute animals and 0.033 g from the 15-minute animals) indicates that the concentration of ACTH in the adrenal was definitely less, on a weight basis, than that in the kidney. Furthermore, the data suggest that the activity in the adrenal was less at 15 than at 5 minutes. An estimate of potency—a very approximate one—indicates that 5 minutes after the injection of 100 μg ACTH per 100 g of body weight, 0.035 g of dry adrenal tissue contains 2 μg of ACTH and that after 15 minutes, 0.033 g contains 0.5 μg of ACTH.

Urine. The urine which had accumulated in the bladder during the 15 minutes following ACTH injection contained no detectable ACTH activity. This is of particular interest because of the large concentration of the hormone in the kidney. Urine from rats in our laboratory is alkaline, a medium in which ACTH is relatively unstable. Rat ACTH incubated in rat urine (pH 8.0) for 15 minutes at 37°C lost ½ to ¾ of its biological potency. In a separate experiment rat ACTH was injected into rats which had been given ammonium chloride so that the pH of the urine was reduced to less than 6. Rat ACTH was demonstrated to be stable in urine at this pH. The urine which had accumulated in the bladder during the 15 minutes following ACTH injection into ammonium chloride-treated rats contained no detectable ACTH activity. It may be concluded that ACTH, under the conditions of our experiments, is not excreted in the urine of rats in a form detectable by the assay method used.

Discussion. The results presented in Table III have been used to calculate the distribution of injected ACTH throughout the organism. These calculations are presented in Table IV. It is to be emphasized that the assay data will allow only approximate estimates to be made. However, there can be no doubt as to the significance of the gross differences which exist between the tissues and body fluids which were analyzed. The studies were conducted in intact rats and hence the possibility must be considered that endogenous ACTH discharge contributed to the quantities found in tissues and blood following injection of exogenous ACTH. Experiments are now being carried out to determine the distribution of injected ACTH in hypophysectomized rats. The rapid disappearance of hormone activity from the blood is not due to destruction of ACTH in this fluid. *In vitro* studies indicate that ACTH is relatively stable in blood at 37°C.

The results demonstrate that the kidney has a remarkable ability to trap injected ACTH. Since rat ACTH was injected into rats it is unlikely that the rapid accumulation of hormone activity in the kidney represents the removal of a foreign protein by this organ. It will be of interest to know whether ACTH, discharged in response to stress, accumulates in the kidney.

No unequivocal data have appeared in the literature regarding endogenous ACTH titers in the blood. However, it is certain that the concentration of ACTH in blood is very low, even under stressful circumstances. In this connection the authors would like to suggest to clinical investigators that the bioassay of kidney rather than of blood for ACTH may provide a useful tool for elucidating the etiology of adrenocortical dysfunction. For example, kidney biopsy could be performed during the course of an exploratory laparotomy for the purpose of determining the status of the adrenals. In hypercorticism with primary involvement of the adrenals, one would expect to find a lower than normal concentration of ACTH in the kidney because of suppression of ACTH release by the high concentration of cortical hormone in the body fluids. In contrast, in hypercorticism secondary to pituitary over-activity, one would expect to find a higher than normal concentration of ACTH in the kidney if it proves to be true that the renal material is of pituitary origin.

Summary. Rat ACTH is a dose equivalent to 100 μg La-1-A per 100 g body weight, injected intravenously into rats, disappears rapidly from the circulating blood of intact and adrenalectomized rats. Five minutes after injection of the hormone into intact rats about 40% of the injected dose is present in extracellular fluid and 20% in the kidneys; after 15 minutes a negligible quantity is present in extracellular fluid and 15% in the kidneys. The adrenals have a relatively high concentration of the hormone although the concentration is lower than in the kidney. It is estimated that less than 1/500th and 1/2000th of the injected dose is present in the adrenals at 5 and 15 minutes after injection, respectively. Liver contains no detectable quantity of the injected ACTH either 5 or 15 minutes after injection of hormone. Injected ACTH could not be detected in the urine in these experiments.

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Observations on a Relationship Between Vitamin B_{12}, Folic Acid and the Citrovorum Factor.* (18688)

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The presence of a growth factor for *Leuconostoc citrovorum* 8081, present in natural products was reported by Sauberlich and Baumann(1). That this factor is closely related to folic acid (PGA) has been established beyond all reasonable doubt. It has been shown that *Leuconostoc citrovorum* factor (LCF) possesses *Streptococcus faecalis* R activity(2,3). Broquist *et al.*(3) have reported that LCF may replace the PGA requirement of the chick. Drysdale *et al.*(4) have observed that the administration of vit. B_{12} enhances the urinary excretion of LCF in

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TABLE I. Effect of Different Levels of Vitamin B_{12} on Liver Storage of PGA in the PGA-deficient Chick.*

Treatment†	PGA‡ γ/g fresh wt	PGA‡ Storage§ equivalent
None	.88	3.26 ± .22
Vit. B_{12} (.1 γ/day)	.92	3.90 ± .25
(.3 ”)	1.41	4.08 ± .08
(.5 ”)	.85	2.85 ± .44
(1.0 ”)	.78	2.32 ± .18
(.3 ” orally)	1.72	4.45 ± .28

* 6 observations per group, including the standard error $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$.

† Injected unless otherwise stated.

‡ Total *S. faecalis* activity.

§ Storage equivalent = γ per liver per 100 g of body weight.

the hyperthyroid rat. In this paper we wish to present certain observations pertaining to the interrelationship of vit. B_{12}, PGA, and LCF.

Experimental. Straight run (New Hampshire ♂♂ X Single Comb White Leghorns ♀♀) crossbred chicks, the progeny of hens fed diet B-1(5) were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing banded and weighed at one day of age. Weights were recorded at weekly intervals. The experimental procedure for Exp. 1 (Table I) was as follows: All birds were placed on a PGA-deficient ration containing sucrose 61 g, alcohol extracted casein 18 g, gelatin 10 g, salts V(6) 6 g, soybean oil 5 g, L-cystine 0.3 g, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, nicotinic acid 5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, choline hydrochloride 150 mg, biotin 0.03 mg, inositol 100 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, and α-tocopherol 0.3 mg. Fortified haliver oil (60,000 U.S.P. units of vit. A, 6000 U.S.P. units of vit. D_3 per g) was given by dropper (2 drops per bird per week). After the chicks had been on this PGA-deficient ration for a 2-week period, the different vit. B_{12} supplements shown in Table I were administered. In Exp. 2 (Table II) all birds were placed on the basal ration containing 200 γ % PGA at 1 day of age. Supplementation was started immediately and continued for 4 weeks. Exp. 3 (Table III) was similar to Exp. 1, except that the supplements were given for the entire 4-week period.

In all cases, 12 chicks were used per group. Crystalline vit. B_{12} was injected into the pectoral muscle using a 1 ml calibrated syringe. Oral administrations were given by pipette. At the termination of the test period, the birds were sacrificed, and the livers removed, frozen immediately, and stored at freezing temperatures until assayed. PGA and vit. B_{12} were extracted and determined by methods previously described(7). The LCF activity was determined on the PGA extract using the medium of Sauberlich and Baumann(1). The procedure used for measuring the conversion of PGA to LCF was similar to that of Nichol *et al.*(8). Liver slices were prepared, using the method of Deutch(9). Samples of 0.5 g,

TABLE II. Effect of Vit. B_{12} on Storage of PGA, Vit. B_{12} and LCF in the Liver of Chicks Receiving Ample PGA.*

Treatment	Vit. B_{12} γ/g	PGA† γ/g	LCF γ/g	$\frac{\text{LCF}}{\text{PGA}}$
None	.17 ± .03	2.26 ± .14	1.67 ± .15	.74
Vit. B_{12} (.5 γ/day inj.)	.29 ± .01	.44 ± .02	1.59 ± .52	3.62
Vit. B_{12} (.5 γ/day orally)	.26 ± .05	4.72 ± .50	2.60 ± .51	.55

* 6 observations per group including the standard error $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$.

All values are on a fresh weight basis.

† Corrected PGA value—observed values minus *S. faecalis* R activity due to the LCF present.

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TABLE III. Effect of Vit. B_{12} on Conversion of PGA to LCF by Liver Slices of PGA-Vit. B_{12} Deficient Chicks.*

		γ LCF per g liver slices fresh wt, present after incubation	
Treatment	Vit. B_{12}, γ/g	PGA, 5 γ/flask	PGA, 5 γ/flask + ascorbic acid, 10 mg/flask
None	.01 ± .01	.54 ± .16	1.35 ± .06
Vit. B_{12}, 1.0 γ/day, inj.	1.02 ± .42	.15 ± .09	1.06 ± .35
Vit. B_{12}, 1.0 γ/day, orally	.83 ± .26	1.01 ± .29	1.84 ± .31

* 5 observations per group, including the standard error $\sqrt{\frac{\Sigma d^2}{n\ (n-1)}}$.

weighed to the nearest mg, were used. In all cases freshly neutralized ascorbic acid was added immediately before the flasks (25 ml Erlenmeyers) were agitated in a constant temperature bath, at 37°C for 2 hours. Duplicate determinations were made in all cases.

Results and discussion. The injection of vit. B_{12} at a level of 0.1 γ or 0.3 γ per day (Table I) increased the PGA stored in the liver, in accordance with previous results(7). However, when the level of vit. B_{12} injected was increased to 0.5 γ per day a depression in the PGA liver storage resulted. The injection of 1.0 γ of vit. B_{12} per day produced a still greater depression of the liver PGA. The oral administration of vit. B_{12} at a level of 0.3 γ per day, produced results similar to those obtained by the injection of 0.3 γ of vit. B_{12} per day.

The second experiment (Table II) differed in that the vit. B_{12} deficient chicks received ample PGA (200 γ %). Under these conditions the injection of 0.5 γ of vit. B_{12} per day resulted in a definite depression of the corrected PGA stored in the liver while no change in the LCF was observed. On the other hand, the oral administration of 0.5 γ of vit. B_{12} per day produced a marked increase of both the LCF and PGA levels as measured by liver storage. The oral administration of 0.5 γ of vit. B_{12} per day produced liver stores of vit. B_{12} slightly lower than those obtained by the injection of 0.5 γ of vit. B_{12} per day. It is of interest to note that although there was no increase in LCF upon the injection of vit. B_{12}, the ratio of LCF to PGA was increased about 6 fold over that of the control group and the group receiving orally administered vit. B_{12}. This indicates that the injected vit. B_{12} displaced the equilibrium between LCF and PGA in favor of LCF. In the case of the oral administration of vit. B_{12} no change in the LCF-PGA ratio was observed. This shows that although there was an increase in both LCF and PGA levels, there was no disruption in the LCF-PGA equilibrium, as in the case of the injected vit. B_{12}.

In Exp. 3, as in Exp. 1, PGA-vit. B_{12} deficient chicks were employed. The administration of vit. B_{12} at the level of 1.0 γ per day produced results similar to those observed in Exp. 2, *i.e.*, the liver levels of vit. B_{12} were similar regardless of the route of administration. Furthermore, the injection of vit. B_{12} resulted in a significant lowering of the liver tissue level of PGA, while the LCF level remained unchanged. The oral administration of vit. B_{12} increased the liver levels of both PGA and LCF. Liver slice studies (Table III) were made on the same birds. The administration of vit. B_{12} by injection significantly decreased the amount of PGA converted to LCF. The addition of ascorbic acid overcame this inhibition. The oral administration of vit. B_{12} significantly increased the amount of PGA converted to LCF either with or in the absence of ascorbic acid.

These data demonstrate that vit. B_{12} plays some role in the conversion of PGA to LCF. Whether this action of vit. B_{12} is that of a specific vitamin action or an indirect action is unknown. The problem is complicated by the action of ascorbic acid, the intricacies of intestinal synthesis and the fact that there is a significant difference in the effect of vit. B_{12} when fed or injected. The action of ascorbic acid does not appear to be specific, since it can be replaced by glucoascorbic acid(8). It must furthermore be remembered that the natural means of receiving nutrients is by absorption from the alimentary canal. It is also well known that the intrinsic factor(9) has a definite effect on the utilization of vit. B_{12}. With these facts in mind, it is possible that the vit. B_{12} absorbed from the tract is altered in such a manner that it may then act as a precursor to a coenzyme participating in an enzyme system which converts PGA to LCF. If this is the case, it is possible that the injected vit. B_{12} is similar enough to the coenzyme to compete for the apoenzyme, thus producing the inhibition noted. However, the effect of ascorbic acid in overcoming this inhibition is more obscure.

Summary. Vit. B_{12} injected at low levels increased the PGA stored in chick liver tissue. Higher levels of injected vit. B_{12} suppressed the liver storage of PGA. Vit. B_{12} administered orally, increased the level of both PGA and LCF as measured by chick liver storage.

Vit. B_{12} injected at the level of 1 γ per day significantly inhibited the conversion of PGA to LCF in PGA-vit. B_{12} deficient chicks. This inhibition was overcome by the *in vitro* addition of ascorbic acid. The oral administration of vit. B_{12} at the level of 1 γ per day significantly increased the conversion of PGA to LCF with or in the absence of ascorbic acid, in the PGA-vit. B_{12} deficient chick.

The possible relationships and interactions of vit. B_{12}, PGA, LCF, and ascorbic acid have been discussed.

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Essential Amino Acids in Mature Human Milk.* (18689)

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The amounts of the essential amino acids in colostrum and transitional milk from healthy nursing mothers were given in a preceding paper(1). This report presents the values for the essential amino acids in whole human milk as consumed by infants of the same group of mothers after lactation was established.

From 61 healthy mothers (47 white, 14 Negro) who were successfully nursing their infants, 147 samples of milk were collected at intervals ranging from 15 to 362 days postpartum. The mothers were 18 to 42 years old and resided in the Detroit area. Five women receiving known diets varying in quantity according to size and appetite but not in quality, provided 18 samples, each sample being a composite of all milk obtained by completely emptying both breasts at regular intervals during 24 hours. Fifty-six women whose diets were self-chosen provided 129 samples, each consisting of the entire amount of milk obtainable from one or both breasts, 4-8 hours after nursing. Samples were usually collected at the mid-morning feeding period, but in some instances were not obtained until early afternoon. Preceding papers have described in detail the method of manual expression used in the collection of samples(2,3), the preparation and storage of samples(4,5),

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* The investigation represented in part by this paper was partially supported by a grant from the Nutrition Foundation.

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TABLE I. Essential Amino Acids in Mature Human Milk.

	Content per 100 ml whole milk											
	Total solids	Total N	Leucine	Isoleucine	Valine	Histidine	Lysine	Methionine	Phenylalanine	Arginine	Threonine	Tryptophan
24-hr samples*	g	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Mean	12.8	204	97	61	74	25	72	12	42	45	54	20
Min.	11.4	175	72	46	48	20	53	10	35	35	43	15
Max.	13.8	240	118	86	114	30	93	16	58	64	64	26
4-8 hr samples†												
Mean	13.2	192	110	70	71	23	76	14	41	45	55	18
Min.	10.8	147	80	48	51	16	53	9	30	28	40	13
Max.	15.8	255	159	102	105	34	104	21	51	61	76	26

* Eighteen samples from 5 women obtained 46 to 316 days postpartum.

† One hundred twenty-nine samples from 56 women obtained 15 to 362 days postpartum.

the hydrolysis procedure, selection of standards, preparation of media, and technics of analysis(1,6).

Lactobacillus plantarum (8014)† was used for determination of leucine, isoleucine, and valine, *Lactobacillus mesenteroides* (8042)† for histidine, lysine, methionine, and phenylalanine, and *Streptococcus fecalis* (8043)† for arginine, threonine, and tryptophan, with simultaneous determinations upon test mixtures of pure amino acids, vitamins, and salts with every series as "knowns," the percentage recovery of the amino acid in the test mixture being calculated for each assay. In addition, a sample of casein and a composite sample of mature human milk were hydrolyzed with each group of samples and assayed simultaneously. An average value was established for each amino acid of casein and human milk. Any assay in which the value for the amino acid content of the controls and duplicate samples failed to check within ±5% was considered doubtful and not included in the results.

Results. The volumes of the 18 individual 24-hour samples of mature milk and their contents of total solids, nitrogen, and the essential amino acids are given in Table I. The volumes of milk secreted in 24 hours varied greatly among the mothers studied and at different intervals postpartum for the same subject. The concentrations of total solids and total nitrogen were not related directly to the volumes of milk secreted. Amino acid concentrations also varied widely among subjects and at different intervals postpartum and differences in concentration did not parallel changes in contents of total solids or volumes of milk secreted.

For one subject (V.G.) 6 24-hour collections of milk obtained during one lactation period demonstrate the biologic variability which must be recognized as normal in human beings. Analytical data for the composition of human milk frequently are based upon analyses of composite samples composed of milk collected from several women, at various stages in lactation, or representing diverse intervals of milk production during the 24 hours. It is evident that such data might represent the average intake of nutriments from breast milk by a group of infants over a length of time, but would not necessarily provide an accurate estimation of the nutritive intake of a group of infants at a given time or of a single infant over any period of nursing.

Mean and range values for nitrogen and the essential amino acids in the 129 samples of milk from 56 mothers also are given in Table I. The values show quite close agreement between the mean concentrations in the 4-8 hour samples and the means for 24-hour collections of milk, supporting the validity of the analyses of the 24-hour collections as representative of the composition of mature milk from healthy nursing mothers.

Discussion. Physiologic trends are best identified from serial observations and over

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† American Type Culture Collection, 2029 M Street, N.W., Washington, D. C.

15 years ago Wilson(7) emphasized that several (longitudinal) observations on the same individual are more valuable in determining physiologic trends than single (cross-sectional) observations on a large number of individuals. For the first time data have been presented in this and the preceding paper(1) which show the patterns of essential amino acid composition in 24-hour collections of colostrum, transitional milk, and mature milk from women of the same group who were successfully nursing their infants. From this group of healthy mothers, the mean values determined for samples collected under highly controlled conditions, prepared, stored, and analyzed under identical conditions with the same microbiological technics, demonstrate that the concentrations of essential amino acids decrease rapidly during the first 10 days postpartum and reach average concentration levels in mature milk less than one-half those of early colostrum. The much higher concentrations of essential amino acids provided by colostrum during the first few days postpartum may be very important in the nourishment of the newborn infant.

The wide variation among women in a survey in which the samples were not procured from the different subjects at corresponding times during lactation‡ provided mean data (Table I) with great variation but without definite trend. However, among the 56 women who provided 4-8 hour samples were 11 who contributed a sample during each of 3 intervals postpartum: 31-68 days; 77-103 days; and 110-157 days. The results from analyses of these samples were tested by the Analysis of Variance. The variance between women was highly significant ($P \geq 99$) in every instance. The variance between intervals postpartum was significant ($95 < P < 99$) for total nitrogen, leucine, isoleucine, phenylalanine, and threonine and highly significant for histidine, lysine, methionine, and tryptophan. Only arginine and valine showed less variance between intervals postpartum than between women, and except for arginine all mean values showed decreases with time postpartum, indicating that the reduction in concentrations of essential amino acids which is rapid during early lactation(1) continues, but at a slower rate, during mature milk production.

Values for amino acids in human milk reported by Beach; Block; Plimmer and Lowndes; and by Williamson were summarized by Macy, Kelly, and Sloan(8). These workers collected pooled samples of milk and isolated the protein fraction, in which the amounts of the amino acids were determined chemically or microbiologically. The values presented in this communication are based on analyses of whole milk and thus total nitrogen includes non-protein nitrogen and amino acid data include the quantities of "free" amino acids present.

The average values for the concentrations of the amino acids in mature milk (Table I) and in colostrum and transitional milk(1) show a definite relationship with respective mean values for total nitrogen. The values for the same woman at different intervals in lactation and those for different subjects showed wide but not parallel variation in both total nitrogen and amino acid contents. An early paper(9) from this laboratory showed that the non-protein nitrogen content of mature human milk may vary from 25.6 to 59.2 mg per 100 ml, which could be a source of significant variation in the data for total nitrogen. Thus, the average values represent compensating variations which obscure important individual differences with respect to infant nutrition.

Summary. 1. Results are presented from analyses of 147 samples of mature human milk from healthy mothers for nitrogen and 10 essential amino acids. The samples consisted of 18 collections from 5 women of all

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milk secreted in 24-hour periods and 129 collections from 56 women of milk secreted in periods of 4-8 hours. Mean values for concentrations of the amino acids in the 2 groups of samples agreed well. 2. Wide individual variations were found among successfully nursing women and at different intervals postpartum. Mean concentrations of nitrogen and the essential amino acids in 24-hour samples of colostrum, transitional, and mature milk showed a general lowering to the levels in mature milk as lactation was established and application of the Analysis of Variance to the data for 11 women, each of whom provided a sample during the same postpartum interval of mature milk production also indicated a gradual reduction in amino acid concentrations.

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Action of *Bacillus cereus* Toxins on Chicken Erythrocytes.*† (18690)

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A preliminary survey of the action of bacterial toxins on cells indicated that the toxins of *Bacillus cereus* had little or no effect on the rate of respiration of chicken erythrocytes(1), dogfish erythrocytes, *Arbacia* or *Asterias* eggs(2). These toxins did, however, have a marked effect on the osmotic behavior of dogfish and chicken erythrocytes(3). The present investigation was undertaken to analyze these changes in more detail. Such a study might help one to understand more fully the way in which these toxins exert their *in vivo* action and might contribute to an understanding of the relationship between the structure of the cell membrane and the passage of non-electrolytes through it.

Materials and methods. General. An isolation of *B. cereus* was made from the laboratory air. The organisms were grown on agar slants and then transferred to proteose peptone broth for incubation at 37.5°C for 36-60 hr. Following filtration, the toxins were stored at 4°C, 95% humidity. Since some of the material was stored for approximately 2 years before it was used, there was considerable variation in the strength of the toxins in different experiments. Consequently, an assay value would have no meaning. It was noted, however, that the qualitative changes in the erythrocytes were the same regardless of the age of the toxins. The older toxins merely had to act on the cells for a longer time before comparable changes were observed.

Chicken and rabbit bloods were obtained by cardiac puncture and heparinized. Sterile technics were employed except during the final 5-10 minutes when measurements were being made and only data obtained from sterile stocks were considered.

Permeability. Using the apparatus previously described(4), rates of hemolysis, shrinking and swelling were measured. The experimental stock solution contained a mixture of erythrocytes and toxins while the control stocks contained cells and broth or

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† The experimental work was performed in the Department of Zoological Sciences, University of Oklahoma, Norman.

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cells and Ringer Locke. These stocks were incubated at 37°C and after various time intervals aliquots (0.05-0.2 cc) were added to 10 cc of the non-electrolyte or salt solution in the chamber of the apparatus. To measure the hemolysis which occurred during swelling or shrinking, the cell suspension was removed after the measurements were made with the photoelectric apparatus, transferred to a centrifuge tube and the cells were thrown down. The amount of hemoglobin in the supernatant fluid was measured at 545 mμ using a Coleman Universal Spectrophotometer. The volumes of the cells were determined by hematocrit measurements obtained using an air turbine and by making cell counts in the manner usually employed in this laboratory.

Lipid analysis. The stock suspensions were centrifuged, the supernatant fluid discarded and the cells hemolyzed. Two methods of determining the lipid content of the cell membranes were used. One was a weight-loss method(5), which consisted essentially of extracting with alcohol-ether a known weight of dried ghosts and determining the loss in weight. The other was a gasometric technic (6) as used by Dziemian(7).

Results. Permeability. In Fig. 1 are presented typical records. Both sets of hemolysis curves show that the cells which have been exposed to the toxins hemolyze more rapidly than do the controls. This might suggest a difference in permeability but the swelling and shrinking curves show no change in permeability. The fact that the deflection of curve 6 is greater than that of curve 5 indicates a slight increase in "fragility". Spectrophotometric measurements showed that 4%§ of the control and 32% of the experimental cells hemolyzed during swelling in 0.3 M glycerol in Ringer Locke. Similar measurements of cells which had been equilibrated in 0.6 M glycerol in Ringer Locke and then shrank in 1.25x Ringer Locke or 2x Ringer Locke showed 5% and 3% hemolysis for controls and 41% and 22% for experimentals respectively. These data as well as hematocrit measurements indicate that the experimental cells swell. In 2 experiments, for example, after 9 hours exposure to the toxins, the control volumes were 73 μ^3 and 74 μ^3 while the experimental volumes were 118 μ^3 and 116 μ^3. After 15 hours the control volumes were essentially the same, 68 μ^3 and 70 μ^3, while it was impossible to obtain hematocrit readings on the experimental cells since about two-thirds of them had swelled to their hemolytic volumes.

It was found that cells which had been in the presence of the toxins a sufficient length of time to make them quite "fragile", as indicated by a large increase in galvanometer deflection during swelling measurements, still were selectively permeable. The relative rates of swelling in ethylene glycol, glycerol, urea, thiourea and erythritol (all in Ringer Locke) were unchanged by this treatment.

Another series of observations showed that rabbit erythrocytes were much less sensitive to the toxins than were the chicken erythrocytes. Whereas a marked change in "fragility" was observed after chicken cells had been exposed to the toxins for 2½ hours, no change in rabbit cells was observed after 4½ hours exposure. Seven hours exposure did make the rabbit erythrocytes quite "fragile" as indicated by the swelling measurments.

Lipid analyses. In Table I the total lipid contents of control and experimental cells are compared. These data indicate that there is no consistent difference between controls and experimentals. In Table II are presented the data on total and free cholesterol contents. Here again, no consistent difference is noted between the controls and experimentals.

Discussion. Apparently these toxins can alter the structural integrity of the cell membrane without causing a loss of lipid (*cf.* Ballentine and Parpart) (8). It seems reason-

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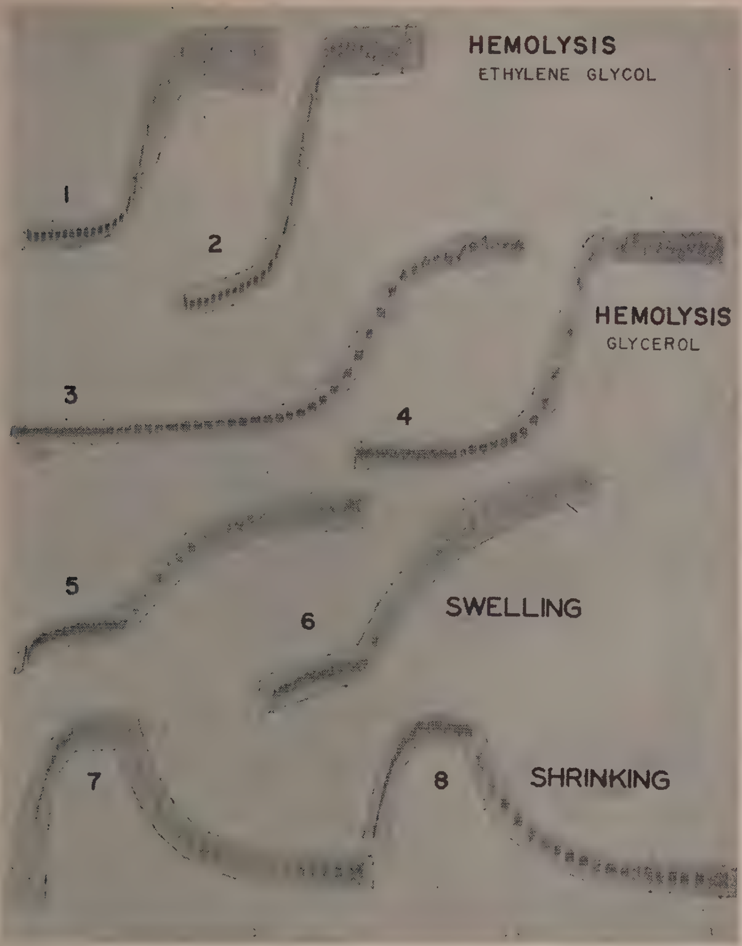

FIG. 1

The osmotic behavior of chicken erythrocytes which have been exposed to the toxins of *Bacillus cereus.* 1—hemolysis of control cells in 0.3 M ethylene glycol; 2—hemolysis of experimental cells in 0.3 M ethylene glycol; 3—hemolysis of control cells in 0.3 M glycerol; 4—hemolysis of experimental cells in 0.3 M glycerol; 5—swelling of control cells in 0.3 M glycerol in Ringer Locke; 6—swelling of experimental cells in 0.3 M glycerol in Ringer Locke; 7—shrinking of control cells in 1.25x Ringer Locke after previous equilibration in 0.6 M glycerol in Ringer Locke; 8—shrinking of experimental cells in 1.25x Ringer Locke after previous equilibration in 0.6 M glycerol in Ringer Locke. Vertical breaks = 1 second, curves 1 and 2; 15 seconds in 3-8, first 15 seconds recorded continuously.

able to suggest that the lecithinase(9) in the toxins is responsible for the changes in the cell surface. As might be expected, the toxins affect the erythrocytes of one species more rapidly than others, the action being most marked with chicken erythrocytes, less with rabbit and least with dogfish.

Apparently the initial action of the toxins can best be described as a "weakening" of the membrane without any marked alteration in the size of the aqueous channels through which small, lipid insoluble non-electrolytes

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TABLE I. Lipid Content of Chicken Erythrocytes Exposed to the Toxins of *Bacillus cereus.*

	Control			Experimental		
Method	% lipid by wt	mg lipid/ 10^9 cells	mg lipid/ cc cells	% lipid by wt	mg lipid/ 10^9 cells	mg lipid/ cc cells
Weight loss	4.1			4.4		
	3.8			3.8		
	3.3			3.3		
	3.9			3.9		
Combustion		.23	2.7		.30	3.1
		.33	3.4		.22	2.7

TABLE II. Cholesterol Content of Chicken Erythrocytes Exposed to the Toxins of *Bacillus cereus.*

	Control		Experimental	
	mg/10^9 cells	mg/cc cells	mg/10^9 cells	mg/cc cells
Total cholesterol	.055	.65	.062	.65
	.060	.61	.052	.62
	.047	.51	.078	.50
	.069	.74	.095	.61
Free ”	.038	.45	.037	.35
	.043	.44	.038	.46

penetrate. Also, there is an increase in the size of the toxin treated cells (*cf.* Wilbrandt) (10). As a consequence of one or both of these changes, the time for hemolysis in isosmotic solutions of lipid insoluble nonelectrolytes is considerably decreased. The increase in "fragility" as indicated by the hemolysis when the cells shrink and then swell might well be a consequence of the structural "weakening" of the membrane. Thus, when the cells go through the mechanical process of shrinking followed by swelling, additional disorientation of the molecules in the membrane superimposed on that due to the action of the toxins occurs and some of the cells hemolyze (*cf.* Hunter) (11). The hemolysis observed when the cells swell first and then shrink is probably a consequence of the increased size of the experimental cells.

Conclusions. 1. Chicken erythrocytes exposed to the toxins of *Bacillus cereus* undergo a change in the surface of the cells which eventually leads to hemolysis. 2. Rabbit erythrocytes similarly treated are much more slowly altered by the toxins, but eventually do show a "fragility" change. 3. There is no significant change in the total lipid, total cholesterol or free cholesterol content of the experimental cells. 4. The experimental cells hemolyze more rapidly in solutions of nonelectrolytes. 5. There is an increase in "fragility" and in the size of the experimental cells. 6. The toxins have no effect on the rate of shrinking of cells placed in 1.25x Ringer Locke following previous equilibration in 0.6 M glycerol in Ringer Locke.

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Oxidative Phosphorylation of Heart Tissue from Vitamin E. Deficient Rabbits.* (18691)

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With regard to the yet unexplained function of vit. E, the suggestion has been made that vit. E may be required in some manner for phosphorylation(1) and that this requirement may be the basis for the increased oxygen uptake of tissues observed in vit. E deficiency(2-4). This suggestion has received some support from observations that minces(5) and water homogenates(6) of dystrophic muscles have a reduced capacity to couple oxidation of substrates with the phosphorylation of creatine. In those investigations, however, the composition of the assay systems apparently limited the efficiency of oxidative phosphorylation as demonstrated by the low ratio of moles of phosphate esterified to atoms of oxygen consumed (P/O ratio) obtained with normal as well as dystrophic tissues. An investigation of the effect of vit. E deficiency on phosphorylations in an assay system normally operating at a high level of phosphorylating efficiency thus appeared desirable. The experiments reported here are the results of such assays on heart tissue preparations from normal and vit. E-deficient rabbits. Rabbit heart was chosen for these experiments since it had been found that high P/O ratios can be obtained with heart preparations(7). In addition, in the rabbit, lack of vit. E adversely affects the heart(8,9) and leads to death from heart failure(10).

Methods. Preparation of animals. Ten young female rabbits weighing 400 to 685 g were divided into 2 groups by equally distributing littermates. They were fed a dystrophy-producing ration designed after that of Basinski and Hummel(11). Half the animals were supplemented orally with 9 mg of synthetic d,l-α-tocopherol (Merck) in 0.15 ml of olive oil each day, 6 days a week. The food was removed 3 hours before supplementation and returned 3 hours afterward, to decrease the destruction of the vitamin in the intestinal tract(12). After 3 to 4 weeks, when the unsupplemented animals were unable to get up by themselves if placed on their sides or back, they were considered ready for the experiment.

Determination of the P/O ratio. Each rabbit was sacrificed by a blow on the head, and the heart was quickly removed and placed in a beaker of ground frozen distilled water. The heart was then cut into 4 or 5 portions, which were blotted and rapidly weighed. After rough mincing, the heart tissue was homogenized in cold buffer(13) in an ice-jacketed Potter-Elvehjem homogenizer. The homogenate was then diluted to 30 ml with cold buffer, and centrifuged at 8500 × gravity for 10 minutes in a room at —5°C. The sediment was resuspended in 30 ml buffer by homogenization, and centrifuged again at 8500 × gravity for 10 minutes. This washing was repeated a

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third time. Finally the sediment was homogenized in a volume of buffer in milliliters corresponding to twice the weight of the whole heart in g. Assays were started immediately after preparation of the final homogenate. The following additions[‡] were made in the order given to the main compartments of Warburg flasks: Water to make a final volume of 3.0 ml, 0.20 ml of .50 *M* potassium chloride, 0.30 ml of 0.10 *M* magnesium chloride, 0.20 ml of .01 *M* adenosine triphosphate (ATP) sodium salt, pH 7.2, .300 ml of .10 *M* potassium phosphate buffer, pH 7.2, .10 ml of 3.5 × 10^{-4} *M* cytochrome C, .30 ml of .050 *M* sodium pyruvate pH 7.2, .10 ml of .050 *M* sodium fumarate, pH 7.2, .10 ml of .50 *M* glucose, .25 ml of yeast hexokinase solution, .10 ml of .50 *M* tris (hydroxymethyl) aminomethane buffer, pH 7.2(17), .30 ml of 0.20 *M* potassium fluoride, and .30 or .50 ml heart homogenate. The center wells contained .20 ml of 20% potassium hydroxide and a strip of filter paper. The flasks were chilled in crushed ice before addition of the homogenate.

TABLE I. Oxidative Phosphorylation of Heart Preparations from Normal and Dystrophic Rabbits.

Assay periods of 15-30 min. were adjusted so that the total oxygen uptakes in all trials were roughly comparable. The observed rate of oxygen uptake was proportional to tissue concentration.

	Volume of homogenate			
	0.30 ml		0.50 ml	
Rabbit	O_2 uptake ml	P/O	O_2 uptake ml	P/O
Normal 1	79 78	2.4, 2.4		
2	68	2.6	68	2.3
3	59	2.6	65	2.6
4	71	2.6	66	2.4
5	68	2.4	78	2.2
Dystrophic 1	64 65	2.7, 2.7		
2	61	2.6	64	2.7
3	51	2.6	65	2.8
4	66	2.5	62	2.4
5	73	2.4	79	2.4

For a single determination of a P/O ratio 2 Warburg flasks with identical contents were incubated at 20°C for 10 minutes. One flask was then rapidly removed from the manometer and treated with 2.0 ml of 17.5% trichloroacetic acid. Oxygen uptake was measured in the other flask for 15 to 30 minutes, and then the reactions were stopped with trichloroacetic acid as described above. After sedimentation of the proteins, the inorganic phosphate of aliquots was determined by the method of Sumner(18). The difference in inorganic phosphate between the first and second flasks gave a direct measure of the phosphate which had been esterified during the oxidation. No correction was applied for possible hydrolysis of phosphate esters during the incubation period. It was necessary to establish that the observed phosphorylation resulted from oxygen uptake and not from glycolysis. The absence of phosphorylation due to glycolysis was shown by the lack of disappearance of inorganic phosphate in flasks in which fumarate was omitted and the system incubated under nitrogen with 15 micromoles of fructose-1,6-diphosphate.

Results. The results of determinations of the P/O ratios of heart preparations from normal and dystrophic rabbits are given in Table I.

These results clearly show that there is no decrease in the ability of dystrophic rabbit heart to couple oxidation and phosphorylation. As stated by Hummel(16), the decreased ability of dystrophic muscle homogenates to phosphorylate creatine is probably a secondary phenomenon related to the marked pathological condition of that tissue in advanced dystrophy. However, the possibility cannot be excluded that vit. E plays some role in these enzymatic processes *in vivo,* which were not detected by our analytical procedures.

[‡] The ATP solution was prepared from Sigma dibarium salt. The cytochrome C (Sigma) was standardized spectrophotometrically(14). The pyruvate was prepared and recrystallized according to Robertson(15). The fumaric acid was recrystallized from water and alcohol, and tris (hydroxymethyl) aminomethane from alcohol. The yeast hexokinase preparation was fraction 3a of Berger, *et al.*(16) in 2% glucose. The bakers' yeast used for hexokinase preparation was kindly supplied by Anheuser-Busch, St. Paul. All inorganic salts were reagent grade, and all solutions were made with glass redistilled water. The solutions of ATP, cytochrome C, pyruvate, fumarate, glucose and hexokinase were kept frozen when not in use.

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It is of interest that with test system used the rate of pyruvate oxidation was increased 60 to 80% by addition of either glucose or hexokinase to the otherwise complete system. This demonstrates that in such preparations substrate oxidation may be controlled by the presence of phosphate acceptors.

Summary. 1. An assay system is described for the direct measurement of the ability of heart preparations to couple esterification of phosphate with oxygen uptake. With this system a high efficiency of phosphorylation is obtained with normal rabbit heart tissue. 2. Heart tissue preparations from rabbits made dystrophic by lack of vit. E showed no decrease in the ability to carry out oxidative phosphorylation.

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Effect of Nitrogen Mustard on Nephrotoxic Nephritis in Rats. (18692)

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The hypothesis that glomerulonephritis is a manifestation of an abnormal immune response(1,2) has led to attempts to alter its characteristics by pharmacological means (3,4). Nitrogen mustard protects rabbits against bovine gamma globulin nephritis and has been reported of value in treatment of the nephrotic stage of human glomerulonephritis (5-7). Since nephrotoxic nephritis in rats is believed to be the consequence of an antigen-antibody reaction(8-10), the effect of nitrogen mustard, which inhibits antibody production(4,11), in preventing the development of this type of nephritis has been studied.

Methods. Male Wistar rats, weighing 60-80 g were used. They were fed dog food cubes and given tap water *ad lib.* Three lots of nephrotoxic anti-rat kidney rabbit serum were prepared by Smadel's method(12), which produced "mild," "moderate," and "severe" nephritis as judged by proteinuria, edema, and survival when 0.1-0.2 ml per 100 g of rat was given intravenously on 3 successive days. Methyl-bis (beta-chloroethyl) amine hydrochloride (HN_2) 0.3-0.4 mg/kg was given intravenously for 4 or 5 successive days beginning 2 or 3 days before the first injection of nephrotoxic serum. The minimal leucocyte depression averaged 67% and usually coincided with the time of administration of the nephrotoxic serum. Urines were collected for 16-hour periods for quantitative protein determinations by the Shevky and Stafford method and for microscopic examination of the sediment. The animals were weighed and examined for evidence of edema

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on alternate days for 2 weeks and then weekly for periods up to 2 months.

Results. Fifty-six rats were divided into 3 groups of 14-22 and given one of the 3 nephrotoxic sera. Approximately half of each group served as controls, the others being treated with HN_2. Survival was influenced by the toxicity of HN_2 as well as by the severity of the induced nephritis. There was no mortality in the control groups given "mild" and "moderate" nephritis, while 6 of 9 rats with "severe" nephritis died in renal failure from 6-14 days after the first injection of nephrotoxic serum. Of 8 rats given HN_2 and "mild" nephrotoxic serum, 5 died apparently from HN_2 intoxication. All 10 rats receiving a slightly smaller dose of HN_2 and "moderate" nephrotoxic serum survived. Four of 11 treated rats with "severe" nephritis died apparently from HN_2 intoxication and 3 from nephritis, leaving only 4 survivors. Edema was noted only in rats with "severe" nephritis. Subcutaneous edema and ascites were manifested within the first week and persisted 3 to 18 days. In 6 of 11 rats pretreated with HN_2 edema was definitely less than in the control group.

The mean protein excretion per 16 hours in 80 control urinalyses on 65 rats was 0.91 ± 0.57 mg. Maximal increase in proteinuria occurred within 4 days of the first injection of nephrotoxic serum in the 3 control groups, and averaged 3.9, 35.8, and 52.5 mg per 16 hours, respectively. In rats with "mild" nephritis the initial 4-fold increase in proteinuria was maintained during the 2-month period of observation. After the initial peak in protein excretion, rats with "moderate" nephritis exhibited a decrease in proteinuria during the first month followed by stabilization at levels 4 to 10 times those of the control period. Proteinuria in the 3 rats surviving the early phase of "severe" nephrotoxic nephritis was not only maintained but increased during the second month. The early increase in proteinuria manifested by the control rats was not observed in the rats pretreated with HN_2. The 8 animals injected with "mild" nephrotoxic serum failed to develop a significant increase in proteinuria until the fourteenth day. After this time, the 3 surviving rats exhibited increases in protein excretion which were slight but did not differ from those of the control animals with nephritis of comparable severity. In the animals pretreated with HN_2 injection of "moderately" potent nephrotoxic serum failed, likewise, to elicit a sudden, maximal increase in urinary protein excretion; rather, there occurred in the 10 rats a gradually increasing proteinuria, which became maximal and equal to that of the untreated animals on the fourteenth day. The urinary protein excretion of the pretreated rats subsequent to that time did not differ from that of the control animals. Similarly, pretreatment with HN_2 altered the degree of proteinuria during the acute phase of "severe" nephritis. Although a marked increase in urinary protein excretion occurred shortly after the injection of serum, it was but 30-50% of that of the control group. Comparison of the treated and untreated groups during the established phase of the "severe" nephritis is difficult because of the smaller number of surviving animals, but the proteinuria of the pretreated animals remained consistently about 50% of their controls. Urinary sediments contained increased numbers of formed elements in all three groups, both treated and controls. Histologic sections of the kidneys of animals dying during the experiments resembled those described by Smadel(13). No differences were apparent in the control and treated groups.

Effect of HN_2 on Chronic Nephrotoxic Nephritis in Rats. In order to study the influence of HN_2 on chronic nephrotoxic nephritis in rats, nitrogen mustards were given intravenously, 0.3 mg per kg per day for 5 days to 8 animals showing a stabilized increase of urinary protein excretion, ranging from 5.4 to 60.6 mg per 16 hours, 3 or 4 months after injection of nephrotoxic serum. A reduction in average proteinuria per 16 hrs occurred in 7 of the 8 rats, varying from +2.0 to –31.9%, with an average depression of –16.0%. There was no apparent relationship between the decrease in average urinary protein per 16 hrs and the intensity of the HN_2 effect in terms

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of maximal leucocyte depression. Changes of the magnitude noted cannot be considered significant in view of the observation that day to day protein excretion in individual rats varied widely and not necessarily in the same direction as the general trend.

Discussion. It is apparent from our experiments that pretreatment of young Wistar rats with nitrogen mustards failed to protect against the development of nephrotoxic nephritis produced by intravenous injection of rabbit antirat kidney serum. In the 2 groups of animals injected with nephrotoxic serum of either "mild" or "moderate" potency both the pretreated animals and those receiving serum alone exhibited approximately the same degree of proteinuria after the first 14 days of the disease. Although the rats pretreated with HN_2 and injected with a nephrotoxic serum capable of producing a "severe" nephritis excreted less protein during the 8 weeks of observation than the untreated rats injected with the same serum, all the surviving animals manifested evidence of chronic nephritis. The small number of animals permits no statistical evaluation of the apparent difference within this group.

Although HN_2 failed to prevent the occurrence of nephritis, some modification of the early phase of the disease was apparent. The delay of approximately 14 days in the onset of marked proteinuria in the pretreated animals suggests that the HN_2 may have interfered with the initial antigen antibody reaction in the kidney in such a fashion as to delay the manifestations of this combination. Nitrogen mustards are reported to have no effect on antigen-antibody reactions *in vitro* (11), but this does not preclude such interference *in vivo*.

Nitrogen mustards are known to exert profound inhibitory effects on the metabolism of various tissues, including renal tissue, of experimental animals(14). It is possible that this property of HN_2 might effect a reduction in proteinuria during the period of its maximal action. This would account also for the delay in onset of increased proteinuria, but it is not consistent with the failure of nitrogen mustard to reduce regularly the abnormal proteinuria of rats with chronic nephritis.

Conclusions. 1. Pretreatment with nitrogen mustards failed to protect rats injected with nephrotoxic serum against the development of acute or chronic nephritis but did appear to modify certain early manifestations of the disease. 2. No consistent effect on the proteinuria of rats with chronic nephritis was produced by the administration of nitrogen mustards.

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Antibiotic Effects of Circulin Against Certain Gram-negative Plant Pathogens. (18693)

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Circulin, a mixture of polypeptide antibiotics produced by *Bacillus circulans* Q-19, (like the polymyxins to which it is closely related) is very active against gram-negative bacteria, both *in vivo* and *in vitro*(1-4).

This note calls attention to the fact that among the organisms inhibited *in vitro* are

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TABLE I. Comparison Between the Inhibitory Effects of Circulin and Streptomycin Against Certain Bacterial Plant Pathogens.

Organism	Strain	Inhibited by µg per ml of		Ratio of inhibiting concentrations
		C*	S*	C/S
Agrobacterium tumefaciens	59	50	100	0.5
Erwinia amylovora	178	3.1	0.2	15.5
E. atroseptica	E-25	6.2	100	0.06
E. carotovora	C-3	3.1	50	0.06
Xanthomonas beticola	72	3.1	50	0.06
X. campestris	W	0.8	50	0.02
X. phaseoli	W	0.4	50	0.01
X. translucens f.sp. *hordei*	P	6.2	0.4	15.5
X. translucens f.sp. *hordei-avenae*	XT-6	12.5	12.5	1.0

* The observations were made after 72 hours incubation. The preparation of circulin sulfate (C) used assayed 5300 units/mg, and contained at least two components, circulins A and B. The streptomycin sulfate (S) used contained 855 units/mg.

also several plant pathogens.* Table I, for illustration, compares the antibiotic activity of circulin and streptomycin, as determined essentially by a method previously described (1). In almost all cases circulin was more effective than was streptomycin. These preliminary data appear sufficiently promising to justify more extensive work in the greenhouse and in the field. Crude broths containing circulin, probably adequate for field trials can be prepared rather easily(1).

Summary. Circulin possesses high *in vitro* activity against several gram-negative plant pathogens.

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* The cultures used in this study were kindly furnished us by Dr. Curt Leben, of the University of Wisconsin, and Dr. W. H. Burkholder, of Cornell University.

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Effects of Modified Human Globin in Normal Human Subjects.* (18694)

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(Introduced by William Dock)

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To assess the value of any substance which is to be administered intravenously for the emergency treatment of shock, it is imperative that its effects on the blood flow through the kidneys of normals be determined. It would be unwise to accentuate the renal ischemia which is known to exist in the shock state. By the same token, any substance which is to be administered as a possible therapeutic measure in diseases known to involve renal functional disturbances, must have its effects on the kidney determined.

It is the purpose of this communication to report the absence of deleterious effects of infusions of human globin on certain renal functions in normals, and also the absence of any effect on the agglutinability of the red blood cells of patients receiving this substance. For unlike gelatin, globin does not make blood typing difficult.

Methods and procedure. In the present study, a group of 6 convalescent patients with no evidence of renal disease were observed. All subjects were tested at least 10 hours after their last meal and hydrated with 500-1000 cc of water by mouth at least 1-2 hours before the start of the procedure. The continuous infusion method for determination of renal clearances was performed. Blanks of urine and blood were drawn prior to the test proper. Control periods, before infusing the globin solution, were obtained. 5% glucose solution was used as the infusion fluid during the control periods. The test periods used lyophilized modified human globin dissolved in 3.5% glucose solution which made the globin solution isotonic. Each 400 cc of the globin solution contained 16 grams of pure globin. The control and globin infusions contained PAH in similar concentrations. Infusion rates were regulated at 4 cc-8 cc per minute in different patients. Concentrations of PAH were determined in plasma and urine by the Bratton-Marshall reaction(1). Urinary excretions of sodium were done employing the flame photometer with lithium as the internal standard. Hematocrit determinations were made on heparinized blood in Wintrobe tubes centrifuged at 2500 rpm for 30 minutes. Blood samples were also drawn for sedimentation rates, typing and cross matching before the procedure was started, during the control periods and at the end of the globin infusion.

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* The globin was supplied through the courtesy of Dr. Gilbert Bayne of Sharp and Dohme, Glenolden, Pa.

Results. Table I lists the results of the various procedures in the patients studied. As will be noted in these normal subjects, 2 of the 6 studied received the globin infusion at a rapid rate (8 cc/minute) and 4 at a slow rate (4 cc/minute). In the 2 subjects receiving globin at a rapid rate there was no change in their urine flow or clearances of PAH, and in one of these there was a slight increase in sodium excretion during the globin infusion as compared to the control levels. In the 4 subjects receiving the globin at a slow rate, 3 of the 4 showed a slight fall in urine flow and only one of these any decrease in clearance of PAH. As regards sodium excretion during slow globin infusion 2 showed no change and 2 a slight increase.

TABLE I. Effects of Globin Infusions.[*]

Name, age, diagnosis	Urine flow, cc/min.	R.P.F. C_{PAH}, cc/min.	Na. excr., mM/min.
F. (F) 24	*7.1*	*473*	*.101*
Parasitic	*6.1*	*577*	*.119*
Infestation	4.3	615	.141
I.R.,[†] 4 cc/min.	2.6	575	.140
Total, 3 g globin	3.1	618	.184
	4.7	411	.205
	3.4	568	.163
G. (M) 33	*9.4*	*784*	*.105*
Pl. Effusion	*10.4*	*940*	*.114*
I.R., 4 cc/min.	*9.0*	*1045*	*.131*
Total, 6 g globin	7.9	1089	.113
	1.3	812	.135
	1.3	810	.127
	1.8	877	.147
	2.6	945	.136
S. (F) 51	*5.5*	*655*	*.072*
Resolving	*3.6*	*573*	*.070*
Pneumonia	*3.6*	*557*	*.079*
I.R., 4 cc/min.	3.6	605	.101
Total, 7 g globin	4.4	436	.099
	3.8	417	.087
	1.3	339	.070
	0.53	358	.072
	0.38	371	.084
	0.40	377	.079
W. (M) 27	*1.3*	*461*	*.086*
Resolving	*1.3*	*402*	*.088*
Pneumonia	3.0	472	.182
I.R., 4 cc/min.	2.8	360	.177
Total, 7.5 g globin	1.6	560	.183
	1.6	504	.168
	1.7	412	.143
	1.1	500	.203
B. (M) 43	*12.2*	*723*	*.190*
Peptic ulcer	*11.8*	*665*	*.167*
I.R., 8 cc/min.	*15.7*	*723*	*.208*
Total, 28 g globin	15.6	730	.219
	12.8	607	.181
	14.1	763	.219
	11.8	659	.172
	11.4	631	.197
	7.8	584	.212
C. (M) 49	*2.9*	*983*	*.202*
Pl. Effusion	*6.6*	*833*	*.174*
I.R., 8 cc/min.	*12.0*	*985*	*.211*
Total, 30 g	13.0	930	.210
globin	8.3	802	.214
	4.5	660	.192
	1.7	730	.253
	2.3	821	.328
	2.9	802	.315

[*] Figures italicized are control periods; others during globin infusion.

[†] Infusion rate.

It is fair to conclude that in normal persons no very striking or remarkable changes in urine flow, renal plasma flow or sodium excretion may be ascribed to the intravenous administration of globin at these rates.

Discussion. It is known that conditions associated with hemoglobinemia and hemoglobinuria have as a concomitant renal effect, a marked reduction in the effective renal

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plasma flow. In normals, when one infuses a solution of hemoglobin renal ischemia is a striking and immediate effect. It has been postulated that perhaps the resulting renal ischemia is brought about through the formation of ferritin by making hemoglobin iron available to the kidney in the presence of hemoglobinemia(2). It is of considerable interest that the iron-free protein, globin which makes up approximately 96% of the hemoglobin of the body has none of the effects noted with hemoglobin, which strikingly decreases urine flow, renal plasma flow and sodium excretion.

As a therapeutic material globin has occasionally some of the renal effects in normal subjects that one encounters with the use of serum albumin(3,4). It is of interest that a very marked proteinuria is apparent shortly after the start of a globin infusion, and it is estimated(5) that as much as 30% of the infused globin may be lost through the kidneys in 24 hours, so that it would require relatively large amounts of globin to produce any significant increase in the protein metabolic pool(6).

It is estimated that when measured at the same concentration 1 g of modified human globin is equivalent osmotically to 2.65 g of normal human albumin. In our series of patients, a diuresis was not evident, and it is possible that the level of the effective circulating blood volume is not critically increased to produce any significant increase in urine flow. There is suggestive evidence(7) that in cirrhosis of the liver, or in cases with a lowered plasma oncotic pressure and a diminished effective circulating blood volume associated with fluid retention, globin may be of distinct value in therapy.

Of importance is the fact that globin does not possess the undesirable effect of substances like gelatin which alter the agglutinability of the red blood cells, thus making difficult typing and cross-matching when a transfusion might be required subsequently. No changes in ease of typing were noted in blood drawn from these cases. No variations in the sedimentation rates were noted to occur after a globin infusion as compared with the controls, which agrees with absence of rouleux formation. The hematocrits remained unchanged with the doses of globin used in these experiments on normals.

Conclusions. 1. The effects of both slow and relatively rapid infusion of modified human globin in 6 human subjects without renal disease were studied. No untoward effects of the infusion were noted. 2. No significant increase in urine flow over control flows was noted. There was no change in the clearances of PAH during the globin infusion as compared with the control levels. There was a slight increase in the rate of excretion of sodium during the globin infusion as compared with control levels in 2 of the 6 patients. 3. No alteration in sedimentation rate and agglutinability of red blood cells was noted as a result of the infusion of globin.

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Failure of Cortisone to Affect Rate of Disappearance of Antibody Protein.* (18695)

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The administration of cortisone or adrenocorticotrophic hormone (ACTH) during immunization results in low levels of circulating antibody(1-5). After immunization has been established, injection of these hormones may be followed within 6 hours(1,4) to 3 days(3) by a diminution of circulating antibody. The low serum antibody levels might have resulted from an increased rate of degradation of antibody protein, from inhibition of synthesis of antibody or from both of these factors. It was the purpose of the present study to determine whether or not there is an increased rate of disappearance of passively administered antibody from the circulation of cortisone-treated animals.

Methods. Antibody globulin. A pool of 650 cc of rabbit serum contained 1,069 mg of antipneumococcus antibody nitrogen to pneumococcus types 6, 9, 11, 13, 15, 17, 22, and 23 as measured by quantitative agglutination method of Heidelberger and Kabat(8). Antibody globulin was concentrated by precipitation with half saturated sodium sulfate (6,7). The precipitate was separated, washed once with one-half saturated sodium sulfate, recentrifuged and then dissolved in distilled water to make a total volume of 160 cc. The resulting solution had a total protein concentration of 9.0% of which 8.9% was globulin (by the Howe method). Four New Zealand rabbits, 2 males and 2 females, weighing about 3,100 g each, were used. Two rabbits, A and E, a male and a female, were given cortisone†, 10 mg intramuscularly daily for 3 days preceding injection of globulin solution, and for subsequent 11 days. The other two rabbits, B and F, were maintained as untreated control animals under otherwise identical conditions. On the fourth day of cortisone administration to rabbits A and E, each of the 4 rabbits received 38 cc of concentrated antibody globulin solution intravenously. The solution was injected in 2 divided portions 10 minutes apart. Twenty minutes after the second injection, 15 cc of blood was collected from the opposite marginal ear vein for determination of antibody concentration in the serum. Subsequent bleedings of 10 cc each were made after 24 hours, 48 hours and 4 days. On the seventh day, 20 cc of blood was removed and, on the tenth day, the animals were exsanguinated. Antibody determinations were performed in duplicate by the quantitative agglutination technic of Heidelberger and Kabat(7,8).

Results. The amounts of antipneumococcal antibody nitrogen remaining at various intervals following injection of antibody solution are presented in Table I and Fig. 1. It is readily apparent that no difference exists in rate of disappearance of antibody protein in the cortisone-treated rabbits. Indeed, the

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TABLE I. Concentration of Antipneumococcal Antibody after Passive Immunization in Normal and Cortisone-Treated Rabbits.

		Antipneumococcal Antibody Nitrogen (mg AbN/ml serum)						Wt change* in 2-wk period
		Days						
Rabbit	Treatment	0	1	2	4	7	10	(g)
A	Cortisone—10 mg daily from	1.04	.69	.52	.44	.36	.18	–180
E	3 days before antibody admin.	.90	.65	.46	.39	.28	.15	–135
B	None	.97	.64	.46	.37	.32	.20	±0
F		1.27	.78	.57	.46	.32	.20	+165

* Rabbits weighed approximately 3100 g 3 days before antibody was injected.

striking similarity of the curves is evident.

Twenty minutes after the administration of the second half of the 38 cc of antibody solution, the rabbits had between 0.90 and 1.27 mg antibody nitrogen (AbN) per ml of serum. During the first 2 days, the rate of disappearance of antibody protein was rapid. This was thought to be due largely to the establishment of a plasma-tissue equilibrium (9). Subsequently the rate of disappearance was slower and more constant, and when the data were plotted (Fig. 1) an approximate straight line relationship was obtained. The amounts of AbN/ml of serum remaining on the fourth, seventh and tenth days are very similar in all animals.

During the 2-week period of cortisone administration, rabbits A and E, with initial weights of 3,050 and 3,125 g, lost 180 and 135 g, respectively. In the same period of time, untreated control animal B, with an initial weight of 3075 g had a transient weight gain and then returned to the initial weight. Control rabbit F, initially 3125 g, steadily gained 165 g. These changes in weight occurred apparently independently of the rate of elimination of antibody.

Discussion. The unaltered rate of disappearance of antibody globulin from circulation of cortisone-treated rabbits is evident from the similarity of the curves in Fig. 1. In addition, there is a parallel between these curves and those constructed from the data of Heidelberger *et al.*(10) and of Bjørneboe(11). In the 8 animals studied independently in 1942(10), 1945(11) and 1950, the rate of disappearance of passively administered antibody is almost identical. From the "straight line" part of the curve in Fig. 1, after the second day, which is admittedly arbitrarily taken, it is possible to calculate the maximum half life of the passively administered antibody protein to be about 6 days for each animal of the current study. A similar rate of degradation may be calculated for antibody globulin from data of the previous studies(10,11).

Unlike passively administered antibody which only "enters into the metabolic reactions which lead to its disappearance"(10) actively formed antibody globulin was found to partake of synthetic and degradation mechanisms in a manner similar to that of other serum proteins(12). Using isotope methods, a half life of about two weeks was found for such *actively* metabolized protein. In humans, a similar half life period for gamma globulins has been found(13). The disappearance rate of passively administered gamma globulin in humans has been measured under abnormal conditions, *i.e.*, 2 cases of hypoproteinemia, one of the "familial idiopathic" variety, and one associated with a lymphoma. A half life of only 3 to 4 days was found(14).

In the current study, the lack of an ap-

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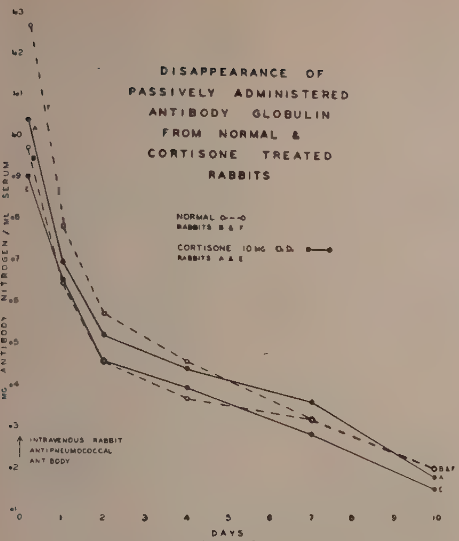

FIG. 1

preciable difference in the disappearance rates of antibody globulin in normal and cortisone-treated animals may be related to several factors. That a distinct loss of nitrogen occurs following the administration of 11-oxygenated adrenal steroids has been well documented(15). However, the mechanism which leads to the nitrogen loss is not well defined. The increased amount of urinary nitrogen may derive from dietary and other mobile sources of nitrogen, *i.e.*, due to an inhibition of the normal rate of synthesis of protein; or it may result from the accelerated breakdown of protein(16-18). The current study was designed to test catabolic rates in the absence of an anabolic effect on the antibody proteins. The results are compatible with the hypothesis that cortisone inhibits synthesis of antibody globulin, since no evidence for accelerated catabolism was found. An objection to this view may be presented. Accelerated protein catabolism may have occurred in the cortisone-treated animals without affecting the passively administered antibody. The globulin, although homologous, may have been handled as a foreign protein and not subjected to the usual catabolic mechanisms since it was not synthesized by the passively immunized rabbit. As another alternative, an inhibition of globulin synthesis may explain the finding of low antibody levels in actively immunized, cortisone-treated rabbits. In this instance, no change would be expected in the rate of disappearance of passively administered protein "tagged" by virtue of its antibody activity. Although it appears somewhat difficult to ascribe the rapid decrease of antibody in actively immunized animals treated with cortisone(1,3,4) to this mechanism, the sustained low antibody levels observed over longer periods of active immunization are probably due in large part to inhibition of globulin synthesis by cortisone (3-5). Morphological studies indicate a disturbance of tissues thought to be associated with globulin formation(3).

In addition to ACTH or cortisone, diminished amounts of circulating antibody in actively immunized animals have been observed following x-ray irradiation(19) and pyridoxine deficiency(20), conditions in which lymphoid atrophy is as pronounced as in hyperadrenalism. In experiments similar to the one reported here, but employing hemagglutinin titers, the rate of disappearance of passively administered antibody was found unaltered in pyridoxin deficient rats(21) or in rabbits treated with x-ray(22). In both studies neither homologous nor heterologous proteins were affected by the procedure employed.

Summary. 1. Four rabbits were given equal amounts of concentrated rabbit antipneumococcal globulin solution. Two of the rabbits were pretreated with cortisone and continued to receive the hormone. During the subsequent ten days, no difference was observed

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from the controls in the rate of disappearance of the "labelled" protein from the circulation. 2. Factors concerned with the interpretation of the data are discussed with reference to the effect of cortisone on protein metabolism.

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Composition of Meconium. Serological Study of Blood-Group-Specific Substances Found in Individual Meconiums.* (18696)

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Blood group substances with the same serological activity as the red blood corpuscles, while demonstrable in various secretions of the body, can not usually be found in feces. However, Witebsky and Satoh(1) mentioned that Yosida(2) had found blood group activity in meconium. Sekimoto(3,4) showed that some samples of human meconium exhibited high activity while others showed little. He separated the infants into secretors and non-secretors and believed that transition types might also be present. Okajima(5) found that the amount of blood group substance diminished rapidly after the first few days of life. Witebsky *et al.*(6) found that a blood-group-destroying-power first appeared in the lower ileum or caecum and that its effectiveness increased in the lower part of the large intestine. Thus in adults, the colon and rectum are free of the blood group activity. It was not determined whether this destroying principle, which was assumed to be an enzyme, is formed in the body or by bacteria growing in the intestinal tract. Blood group substances have also been demonstrated in saliva. However Schiff and Sasaki(7) found that some individuals do not possess the ability to secrete blood group substances. The ability to secrete these substances is hereditary and transmitted as a simple Mendelian dominant. The finding of large amounts of blood group substances in human meconium made it of great interest to determine whether their presence in meconium corresponded with the occurrence of blood group activity in saliva. Also, in view of the conflicting evidence(8,9) concerning the correspondence of the blood group activity of amniotic fluid and the blood type of the infant, comparative studies in this direction, with simultaneous determination of the activity of meconium, were undertaken.

The present report elaborates on the preliminary serological investigations of the blood-group-specific substances found in human meconium(10), while the chemical nature of meconium will be reported elsewhere (11).

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* Data for this paper are taken from a dissertation submitted to the Faculty of the Graduate School of the University of Cincinnati by Dorothy Jean Buchanan, June, 1951, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Experimental. Methods. The blood samples were collected from the infant's heel, defibrinated, and typed with standard anti-A and anti-B serum, according to the usual procedures(12). The samples of saliva were collected on a sterile swab, which was immediately immersed in one cc of normal saline and heated for 5 minutes on a boiling water bath to destroy the enzymes which inactivate the blood-group-specific substances(13). The meconium samples were suspended in 3 times their weight of water. The presence of A and B blood group activity in the saliva and meconium was tested by the method of inhibition of agglutination as described by Morgan and King(14). Standard human anti-A and anti-B sera, which gave maximum agglutination at dilutions of 1:64 with normal saline, were diluted 1:8 for these determinations. 0.1 cc of the material to be tested was allowed to stand for an hour at room temperature with 0.1 cc of the diluted serum. 0.1 cc of a freshly prepared 0.5% suspension of washed A or B red cells in saline was then added. After 2 hours the amount of agglutination was determined macro- and microscopically. Absence of agglutination indicated the presence of active material. A sample of human anti-O serum was obtained from the Blood Grouping Laboratory, Boston, Mass. 0.1 cc of a 1:8 dilution of this serum with albumin (Armour, 0.3 g per cc) was allowed to stand with the sample to be tested for an hour at 4°C. A freshly prepared 0.5% suspension of type O red blood cells in 1:1 normal saline:albumin was added. After 2 hours, the amount of agglutination was determined.

Results. Blood Group Activity in Saliva, Meconium, and Blood.

Table I shows the comparison and correlation of the blood-group-specific substances found in the blood, saliva, and meconium of the infant.

TABLE I.
Comparison of Blood Group Substances Found in Blood, Saliva, and Meconium of the Same Infant.

No. of infants	Blood type	Saliva activity	Meconium activity*	Classification
16	A	A	$1:10^4$—$1:10^7$ A	A S†
4	A	——	——	A N
2	AB	A, B	$1:10^2$—$1:10^3$ A $1:10^3$B	AB S
7	B	B	$1:10^4$—$1:>10^7$ B	B S
3	B	——	——	B N
11	O	O	$1:10^2$—$1:10^3$ O	O S
8	O	——	——	O N
2	O	——	$1:10^2$ O	?

* The meconium was originally suspended in 3 times its weight of water. These figures represent approximate dilution of suspensions at which agglutination first appeared.

† S = secretors; N = non-secretors.

TABLE II. Summary of Blood-Group-Specific Substances in Meconium.

Classification	No.	%	
A secretors	16	22.9	28.6
A non-secretors	4	5.7	
AB secretors	2	2.8	2.8
B secretors	7	10.0	14.3
B non-secretors	3	4.3	
O secretors	11	——	54.3
O non-secretors	8	——	
O ?	2	——	
O not analyzed	17	——	
Total	70	——	100.0

A summary of the blood-group-specific substances found in meconium is shown in Table II. The total per cent figures indicate that the distribution of the various blood types can be correlated fairly satisfactorily with the average of those found in the United States. Wiener(15) states that the distribution of the various blood groups in the United States is as follows: 41% belong to type A, 4% to type AB, 10% to type B, and 45% to blood type O. In all cases except 2 the meconium possessed the same blood group activity as the saliva of the same baby. In all instances the activity found in the meconium corresponded with the blood type of the infant. The A and B determinations were all definite, except in the case of the two infants of type AB. The agglutinations of the blood of these infants formed slowly with anti-A serum, so possibly they belonged to

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TABLE III. Correlation of the Blood-Group-Specific Activity in Amniotic Fluid and Meconium.

Name	Classification of		Activity of	
	Mother	Baby	Amniotic Fluid	Meconium
S	AB secretor	B secretor	B	B
M	A non-secretor	O	No A*	No A
Sh	B secretor	O	No B	No B
H	B ”	O	No B*	No B
W	B ”	O	No B*	No B

* Artificially collected by a syringe from unbroken membranes.

a subgroup of A. This would also explain the low amount of blood group activity A in the meconium of these individuals.

The O activity was much lower in all cases. This may have been the result of the weak antigenicity of the blood type O specific substance. Generally any indication of O activity in the saliva was accompanied by some activity in the meconium. In two cases, the meconium possessed O activity, but there was no indication of activity in the saliva. It is possible that the tests as employed were not sufficiently sensitive to demonstrate the small amounts present in the saliva.

Tests for M, N, and Rh activity. Because meconium had proved such a rich source of the four main blood types, A, B, AB, and O, it was tested for M, N, and Rh activity. The meconiums of 6 infants, whose M and N blood type was known, were tested for M and N activity. No indication of activity was seen. These negative results correspond with the fact that M and N antigens appear to be restricted to the erythrocytes in normal individuals. In several instances, using the general procedure employed by Witebsky(16) for the detection of Rh activity in amniotic fluid, definite inhibition of agglutination occurred. However further work needs to be done to confirm these results.

Blood group activity of amniotic fluid and meconium. One of the classical theories of meconium formation is that it is the accumulation of fetal swallowing of amniotic fluid and products of endogenous formation. Amniotic fluid has also been shown to contain blood-group-specific substances. Putkonen(8) in 1930 stated that these substances corresponded to the blood type of the baby and not of the mother; however Kabat *et al.*(9) reported a case in which the type of substance in the amniotic fluid was A, corresponding to the blood type of the mother rather than to that of the infant, which belonged to the blood group O. In order to study these relationships further the amniotic fluid from a series of deliveries was collected. The blood and saliva of both mother and infant, and the meconium, were also obtained. In a series of 30 deliveries, 5 cases were found in which the mother had either A, B, or AB blood type and the baby a different blood type. The results on the saliva, meconium, and amniotic fluid are presented in Table III.

The results confirmed the work of Putkonen in that none of the blood-group-specific substances characteristic of the mother were found in the amniotic fluid. Whether the blood group substances in meconium resulted from an accumulation of swallowed amniotic fluid or whether the meconium was the source of the blood group substances in amniotic fluid was not determined.

The availability of a large and easily accessible source of human blood group substances in meconium obtained from human infants is of great theoretical and practical interest. It affords an unlimited supply of hitherto practically inaccessible blood group substances for serological and chemical study. It will also allow a comparison of the substances from human and animal sources.

Meconium also offers a great promise in blood transfusion as a source of neutralizing agents for agglutinins in pooled plasma or blood, since it is a very potent source of blood-group-specific substances which can be simply purified, and should be free of anaphy-

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lactic properties to humans.

Summary. 1. The blood-group-specific substances present in meconium from a series of 53 infants were found to correspond to the blood type of the baby and its ability to secrete blood group material in its saliva in all except 2 cases in which O activity was demonstrated in the meconium but could not be found in the saliva. 2. The meconiums of blood groups A and B were more active than those of groups AB or O. 3. No M or N activity could be demonstrated in meconium. 4. The blood-group-specific substances in amniotic fluid corresponded with the blood type of the baby and not that of the mother.

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Excitation of the Isolated Ventricular Septum of the Heart. (18697)

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One of the basic assumptions traditional in electrocardiographic interpretation is that the excitatory process in the ventricular septum starts symmetrically on either side and progresses toward its center. The potentials produced in the ventricular septum might then so neutralize one another that the manifest electromotive force recorded at a distance would be negligible. This hypothesis of ventricular septal excitation is most useful in the explanation of many electrocardiographic phenomena, but it has not been tested experimentally in any thorough manner.

In the dog's heart the arrangement of the coronary arteries includes a large septal branch which arises near the origin of the left coronary artery and passes directly into the musculature of the septum. With such an arterial supply insured, attempts were invited to perfuse the heart by the Langendorff technic with the free walls of the right and left ventricles removed. Under such circumstances the surfaces of the right and left side of the septum could be explored by an electrode under direct vision and the electrical activity recorded.

Methods. The heart was suspended from a cannula, tied in the aorta, through which the perfusate was introduced under pressure. Tyrode's solution with 1% added glucose was used routinely, though in the latter experiments approximately 100 cc of centrifuged erythrocytes were added to the total volume of perfusate (usually 2,000 cc). The lower one third to one half of the organ was submerged in the perfusate in a container beneath the heart. The distant electrode was placed in a corner of the container at a distance of approximately 15 cm from the heart. The reference tracing was uniformly obtained by recording the potentials between a wire electrode about the aorta and adjacent atria and a distant electrode. Relative positivity of the aortic electrode was represented by an upward deflection on the finished record. After stabilization of the heart rate the free walls of the right or left ventricle or both were excised nearly up to the atrioventricular groove. Routinely a band of ventricular muscle remained along the atrioventricular sulcus. This ridge of muscle was also removed in the later part of 2 experiments without alteration of the pattern of the reference tracing. The exploration of the septal surfaces was carried out by 3 technics: (1) by a bipolar method, in which the points from which the electrograms were obtained were approximately 1 mm distant from one another; (2) by a unipolar method in which a single electrode was used to explore the septal surface, and the potentials between it and the distant electrode were recorded; (3) by a bipolar method of recording wherein two electrodes were placed on opposing sides of the septum. All the utilizable records were obtained within one-half hour after the removal of the heart from the animal.

The recording equipment was a Sanborn

Tribeam electrocardiograph locally modified. The responses of the two electrocardiographic recording systems were similar. The response to a 1 mv sine wave input began to decrease appreciably after 30 cycles/sec.; at 40 cycles /sec. it was 75%; and at 75 cycles/sec. it was 60% of the DC response. The camera speed was 75 mm/sec. and the standardization was 5 mv = 1 cm. Measurements were made to the nearest 0.25 mm using a 0.5 mm grid and a hand lens. The measurements were then converted to time and reported to the nearest 0.001 sec. (1 mm = 0.0133 sec.).

Results. Perfusion of the heart was initially successful in 15 experiments, but in only 6 were results sufficiently adequate for analysis. In the others either the early development of fibrillation prevented adequate records or there was absence of normal sinus conducted beats. In many of the preparations nodal rhythm appeared as a stable and inconvertible mechanism, and in others there was partial or complete heart block. Measurements were made on those records where such abnormalities had not appeared.

The first observation was the consistent finding that the potentials of the isolated septum were such that the manifest vector was of considerable voltage and oriented positive toward the base and negative toward the apex. No shortening of the QRS duration occurred in any experiment after removal of the free walls of both ventricles.

The second finding was that in all preparations there was evidence that predominantly the left side of the septum was activated early and that the arrival of the excitatory process was relatively delayed and more irregularly deployed over the right side of the septum. These results in the first experiments were confusing in that it appeared that the hearts were showing spontaneous right bundle-branch block. The first part of the right side of the ventricular surface to be excited was either the middle or the lowermost portion. The upper part of the right side of the septum was activated relatively late, and in one experiment the upper left side was relatively late in its activation. The septal side of the conus of the right ventricle varied in its time of activation in various hearts, activation usually being relatively early.

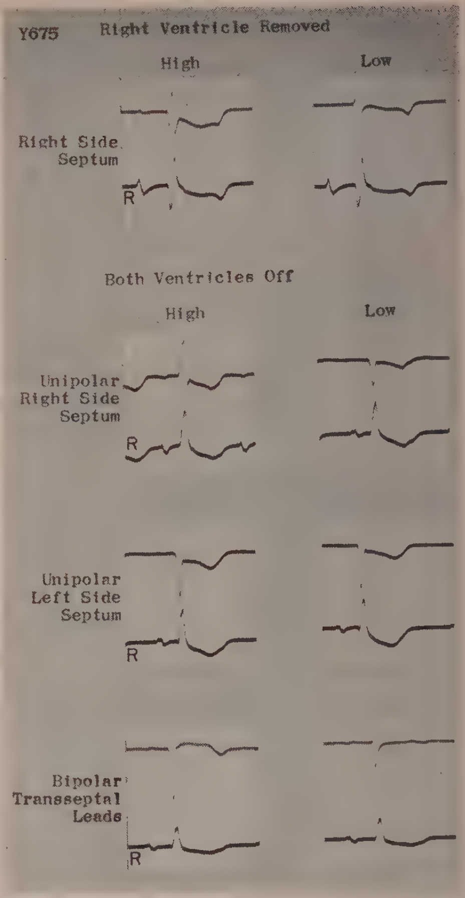

FIG. 1
Electrograms of the perfused septum, explained in text.

A third finding was that it was impossible to obtain exact reproducibility of results over a period of time during which electrodes were removed and replaced on areas believed to be the same by visual inspection. In repeated tests in the same preparation the times of arrival of the excitatory state varied in one instance 0.010 second on the right side of the septum in an area which if not the same must have been closely adjacent. An illustration of the type of records obtained is given in Fig. 1, and the times of septal excitation in three experiments are given in Table I.

TABLE I. Ventricular Septal Activation: Bipolar Exploration: Time of Deflection in Exploratory Electrogram from Onset of QRS in Reference Tracing.

	Animal Y37		Animal Y675		Animal Y683
	Right	Left	Right	Left	Right
Conus area			.018		.016
High	.034	.025	.034	–.007	.013
Mid	.002	–.003	.020	–.010	.030
Low	.001	.009	.011	–.007	.012

A fourth finding was that an incision into the septum calculated to produce right bundle-branch block caused delay in the excitation reaching the tricuspid (upper) and conus portions of the septum, with little change in the time of excitation of the apical area.

Comment. The observations recorded indicate that the excitation of the ventricular septum is a more complicated process than a simple perpendicular spread from the opposing endocardial surfaces. The possible criticism that the hearts in these experiments were not in sufficiently good physiologic state for the data to be applied to the normal animal can be answered only by stating that sinus rhythm, normal atrioventricular and ventricular conduction were present and the ventricular mass contracted vigorously. It might be contended that in the excision of the right ventricle there was accidental damage to the right bundle branch. Every effort was made to avoid injury to the septum and none was apparent after the free wall had been excised. The experiments indicate a variability in the patterns of excitation of the ventricular septum from animal to animal. The constant pattern of the bipolar electrogram of the isolated septum with the positive end of the vector toward the base of the heart can best be explained by the excitatory process spreading away from the apex in at least a large number of its component parts.

The results obtained in our experiments are not without support from the early workers in the field. To quote Lewis and Rothschild(1), "Our readings for the right side of the septum are variable 0.0033 and 0.0060 for the mid-septal area and 0.0243 and 0.0144 for the high septal region." They explained the late activation of the high right septal surface by a lack of Purkinje fibers in this area. Recent workers, as Kossmann and associates(2), have encountered large moderately late electromotive forces in the outflow tract of the right ventricle and have suggested that these might be related to excitation of a band of right ventricular muscle called the crista ventricularis.

Pruitt and associates(3) have reported investigations indicating that the excitatory process in ventricular muscle travels more rapidly when spreading in the direction of the predominant long axis of the fibers. It seems pertinent in this regard that some of the superficial muscle fibers of the upper left septum spiral deeply in the septum to appear near the endocardium of the apex of the right ventricle. Clinically, the fact that the electrocardiographic picture of right bundle-branch block is quite commonly associated with anteroseptal infarction is of interest(4) and may possibly be related to a predominant left-to-right septal activation in man.

Conclusions. 1. It is possible to obtain electrograms on the isolated perfused dog's ventricular septum, and these show a reproducible pattern. 2. The records indicate that the excitatory process in these experiments traveled in a complicated fashion and the pattern varied somewhat from animal to animal. In some of the experiments the excitatory pathways varied over a period of time. As a basic rule, the left side of the septum was found to be activated early and the upper right side late. 3. The apparent absence of septal electromotive forces in electrocardiograms recorded by usual clinical methods, which has been explained by neutralization of potentials spreading inward from each side, is not supported by these preliminary observations on the isolated septum.

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Relationship Between Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses. (18698)

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It was recently decided to investigate the relationships of epidemic keratoconjunctivitis virus to certain other viruses. With this plan in mind a strain of virus was secured from Dr. F. S. Cheever in Boston the usual intracerebral titers of which were reported to be approximately 10^{-6} in mice(1-3). Titers obtained in our laboratory were close to 10^{-9}. The virus was neutralized by known St. Louis encephalitis sera and to a lesser degree by Japanese B and West Nile encephalitis sera. These results were fully confirmed by Dr. Cheever(4). Subsequently a strain of keratoconjunctivitis virus obtained from Dr. Alson E. Braley in New York gave the same high titer in mice. This strain was sent to Dr. Cheever who found that it was also neutralized by St. Louis encephalitis serum. Since laboratory contamination was ruled out by these results, tests were set up which established a close immunological relationship between the two strains of epidemic keratoconjunctivitis virus and the virus of St. Louis encephalitis.

Materials and methods. Epidemic keratoconjunctivitis (EK) viruses. Two strains of the virus were employed in these tests. The one (labeled EK-TC17) obtained in 1950 from Dr. Cheever then at Boston was designated the *Cheever* strain. It had been frozen since 1947 and had originally come from Dr. Braley in New York. The other (labeled EK103-p.61) was obtained directly from Dr. Braley in 1950. It had been apparently through more than 100 mouse passages and had been stored at various times either in a mechanical deep freeze or in a solid carbon dioxide cabinet. It was designated the *Braley* strain. Both strains of virus were, in all probability, derived from the same pool of material frozen in 1942 by Dr. Murray Sanders in New York.

Other viruses. Two strains of St. Louis encephalitis virus (SLE) were used. The one, designated *Webster*, had been kept in the lyophilized state since 1943. It was obtained from Dr. A. B. Sabin in Cincinnati and was presumably the strain isolated in 1933 by the late Dr. L. T. Webster of the Rockefeller Institute, New York. The other designated *Hammon* was obtained in 1950 from Dr. W. McD. Hammon in Pittsburgh. Both viruses had been through numerous mouse passages and were supposedly derived from the *Webster Number 3* strain. The Nakayama strain of Japanese B encephalitis virus (Jap B) had been lyophilized in 1943 and was obtained from Dr. Sabin. The West Nile encephalitis virus lyophilized in 1946 was supplied in 1943 by Dr. Joel Warren, Washington, D. C.

Virus suspensions and titrations. Several infected mouse brains were ground in a mortar with alundum and enough undiluted normal rabbit serum to make a 20% suspension. After light centrifugation the supernatant fluid was either used the same day or stored until needed in solid carbon dioxide in approximately one cc amounts in rubber stoppered vials. Titrations of each suspension were performed by making serial tenfold dilutions in saline solution containing 10% normal rabbit serum (rabbit serum-saline) and inoculating them intracerebrally into groups of mice. The normal rabbit serum had been heated at 56°C for one half hour. LD_{50} titers were determined according to the method of Reed and Muench(5).

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1. Sanders, M. and Alexander, R. C., *J. Exp. Med.*, 1943, v77, 71.

2. Braley, A. E., *Trans. Am. Acad. Ophth. and Otolaryng.*, 1944, Jan.-Feb.

3. Heyl, J. T., Allen, H. F. and Cheever, F. S., *J. Immunol.*, 1948, v60, 37.

4. Cheever, F. S., Proc. Soc. Exp. Biol. and Med., 1951, v77, 125.

5. Reed, L. J. and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

Table I. Neutralization Tests with Epidemic Keratoconjunctivitis Virus (Braley Strain). Protocol

Serum in test	Dilutions of virus in mixture*									LD_{50} titer	Neutralization index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Normal rabbit	—	—	—	—	4/4†	4/4	3/4	0/4	0/4	8.3	—
SLE hyperimm. rabbit	4/4	1/4	0/4	0/3	—	—	—	—	—	2.7	400000
Jap B ” ”	—	—	4/4	2/4	1/4	0/4	—	—	—	5.2	1200
Jap B human‡	—	—	—	4/4	4/4	3/4	1/4	—	—	7.5	6

* Serum-virus mixtures incubated 2 hr in water bath at 37°C and then .03 cc inoculated intracer. into groups of mice.

† Numerator shows No. of mice died; denominator, No. inoculated.

‡ Presumable inapparent laboratory infection.

Neutralization tests. In the neutralization test usually employed, decimal dilutions of virus were added to equal amounts (0.15 cc) of the undiluted serum to be tested. The mixtures were incubated in a water bath at 37°C for 2 hours and then inoculated intracerebrally (0.03 cc) into groups of mice. Deaths were recorded daily and the resultant neutralization indexes were calculated according to the method outlined in Diagnostic Procedures for Virus and Rickettsial Diseases (6). Heated normal rabbit serum was used as a control. A neutralization test was also used in which dilutions of serum were mixed with a constant quantity of virus. Serial four-fold dilutions of the test serum were made in normal rabbit serum and distributed to tubes. Equal amounts (0.15 cc) of the virus dilution selected were added to each tube and after incubation at 37°C for 2 hours the mixtures were inoculated into groups of mice. Normal rabbit serum was used as a control. After the two-hour incubation period the control mixture was serially diluted in rabbit serum-saline and inoculated intracerebrally into mice in order to determine the number of LD_{50} doses present in the mixture.

Sera. Most of the sera were kept in 'lusteroid' tubes or rubber stoppered vials at –30°C in a mechanical deep freeze. Some sera which had been prepared in the past were lyophilized or were stored in the liquid state in the refrigerator.

Animals. The mice were albinos of either sex obtained from a local dealer but bred in our own laboratory for several years. They were between 18 and 24 days old when used and weighed approximately 8 to 11 g. All other animals were obtained from local dealers. The guinea pigs were of both sexes and weighed between 300 and 700 g. Rabbits also were of either sex and weighed between 2 and 3 kg. Albino rats were young male adults weighing approximately 150 g.

Results of neutralization tests. The various neutralization tests were carried out in which undiluted serum and serial dilutions of virus were mixed. The protocol of one test is given in Table I. The neutralization indexes of this and other experiments are summarized in Table II. It is apparent from the data that when the neutralization test is used, serologic distinction of the EK viruses from the SLE viruses is difficult, if not impossible. Most of the sera from EK convalescent animals neutralized the EK and SLE viruses to an equal degree. However there were two exceptions. In one case the pool of serum from the guinea pigs neutralized the homologous virus but not the heterologous virus and in another case the reverse occurred. The serum of Rabbit 1, convalescent from the first intracerebral inoculation neutralized the heterologous SLE virus but not the homologous EK virus. No tests were performed with EK sera against the Jap B and West Nile viruses. The SLE sera obtained from convalescent or hyperimmune animals also demonstrated equal neutralizing ability against both EK and SLE viruses. In addition it was found that the SLE hyperimmune sera neutralized to a lesser but significant degree the related Jap B and West Nile viruses. SLE convalescent

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6. *Diagnostic Procedures for Virus and Rickettsial Diseases,* New York, Am. Public Health Assn. 1948, p200.

TABLE II. Results of Cross-Neutralization Tests.
Summary

Sera in test	Virus used in neutralization test: EK Cheever	EK Braley	SLE Webster #3	SLE Hammon	Jap B	West Nile
EK (Braley)						
Rabbit 1 i.cer. conval. (1st)	—	4	320	—	—	—
” 1 ” ” (2nd)	—	600	1000	—	—	—
” 9 i.perit. ”	—	6000	10000	—	—	—
” 10 ” ”	—	<100	40	—	—	—
Rat pool i.cer. ”	—	200	2000	—	—	—
G. pig pool i.cer. ”	—	100	3	—	—	—
SLE (Webster #3)						
Rabbit pool hyperimmune	100000	400000	>100000	160000	40000	10000
” ” ” *	20000	—	12000	10000	320	1000
” ” i.cer. conval.	—	6000	4000	—	—	—
” ” ” ” †	—	40000	—	—	—	—
G. pig pool ” ”	—	3000	2000	—	—	—
Jap B						
Rabbit pool hyperimmune	2500	1200	150	1600	100000	1000
Monkey i.cer. conval.‡	1600	—	<15	500	63000§	—
Human‖	2	6	2	3	12000	60
West Nile						
Rabbit pool hyperimmune*	3000	400	3000	1600	<20	15000
Herpes simplex— rabbit—hyperimmune	—	<6	—	—	—	—
Mengo encephalomyelitis—rabbit*	1	<6	—	—	—	—
Western equine encephalitis—rabbit	1	<10	—	—	—	—
Eastern ” ” ”	1	<10	—	—	—	—

* Kindly provided by Dr. Joel Warren, Washington, D.C.
† Hammon strain of St. Louis encephalitis virus.
‡ Kindly provided by Dr. Albert B. Sabin, Cincinnati.
§ Index furnished by Dr. Albert B. Sabin.
‖ Presumable inapparent laboratory infection.

sera were not tested against these last two viruses.

Jap B sera also yielded interesting results. The hyperimmune rabbit serum showed a high index against its own Jap B virus but with it there occurred considerable cross-neutralization against the EK, SLE and West Nile viruses. The results with a Jap B monkey convalescent serum were practically the same. In each case however the homologous index was considerably higher than the heterologous indexes. The human Jap B serum, which neutralized Jap B virus well, showed no significant neutralization of the EK and SLE viruses but did show some neutralization against the West Nile virus. The West Nile serum neutralized the West Nile, EK and SLE viruses but again the index was highest against the homologous West Nile virus. There was little neutralization of the Jap B virus. Sera prepared against the viruses of Herpes simplex, Mengo encephalomyelitis and equine encephalomyelitis (Eastern and Western strains) failed to neutralize both strains of the EK virus.

Neutralization of rabbit passage EK virus. Early in the course of the investigation it was noted that intracerebral inoculation of EK virus into rabbits caused a fatal encephalitis. After 3 intracerebral passages in rabbits followed by three intracerebral passages through mice the virus was used in a neutralization test. After preliminary titration had revealed the same high titers in mice, the dilutions for the test were selected as before. The results are shown in Table III. The SLE hyperimmune serum neutralized the EK virus with an index of 200,000 while the Jap B and West Nile hyperimmune sera yielded indexes of 600. There was no EK hyperimmune serum available but a rabbit convalescent serum gave an index of 3000. These indexes and those obtained with the sera from the same animals in the test against the

TABLE III.
Neutralization of Epidemic Keratoconjunctivitis (Braley Strain) Rabbit Passage Virus.

Rabbit serum in test	Dilutions of virus in mixture 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50} titer	Neutralization index
Normal	—	—	—	—	—	4/4	2/4	0/4	8.0	—
SLE hyperimmune	4/4	1/4	0/4	0/4	—	—	—	—	2.7	200000
EK (Braley)—i.cer. conval.	—	4/4	4/4	1/4	0/4	—	—	—	4.7	3000
Jap B hyperimmune	—	—	3/4	3/4	0/4	0/4	—	—	5.2	600
West Nile "	—	4/4	3/4	3/4	0/4	—	—	—	5.2	600

TABLE IV. Cross-Neutralization Tests with Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses Using Dilutions of Serum and Constant Quantity of Virus.

Virus in mixture	Serum used	Dilution of serum used Undil.	1:4	1:16	1:64	1:256	1:1024	50% protective end-point of diluted serum
EK 160 LD_{50} doses	EK rabbit conval.	0/5	4/5	4/5	5/5	5/5	5/5	1:3
	SLE " "	0/5	0/5	1/5	1/5	5/5	4/5	1:105
	Normal rabbit control	5/5	—	—	—	—	—	—
SLE 200 LD_{50} doses	EK rabbit conval.	0/5	5/5	5/5	5/5	4/5	5/5	1:2
	SLE " "	0/5	1/5	5/5	5/5	5/5	5/5	1:7
	Normal rabbit control	5/5	—	—	—	—	—	—

The EK rabbit serum showed neutralization index of 600 against EK virus and 1000 against SLE virus; the SLE rabbit serum showed index of 6000 against EK virus and 4000 against SLE virus.

mouse passage virus were practically identical. The inferences that can be drawn from this experiment are (1) the presence of another virus was not revealed and (2) rabbit passage did not disclose any change in the virus.

Neutralization tests employing dilutions of serum and a constant amount of virus. After it became apparent that the conventional neutralization tests were inadequate to differentiate EK from SLE virus, it seemed desirable to explore the test in which the virus was kept constant but the serum was diluted. The sera from convalescent rabbits with neutralization indexes that were approximately equal against both EK and SLE viruses were used. The serum dilutions were tested against 160 LD_{50} doses of EK virus and 200 LD_{50} doses of SLE virus and the results are given in Table IV. The EK serum gave a 50% protective end-point of 1:3 against the homologous EK virus and a 1:2 end-point against the heterologous SLE virus whereas the 50% end-point of the SLE serum was 1:105 against the heterologous EK virus and 1:7 against the homologous SLE virus. In both tests the SLE serum was more potent regardless of the virus used thus indicating that this method of doing the neutralization test did not serve to differentiate the two viruses. Further tests are in progress.

Infectivity tests in animals. The tests revealed the only marked differences between the EK and laboratory adapted strains of SLE virus. Most of the experiments were done with the *Braley* strain of EK virus but essentially the same results were obtained on a more limited scale with the *Cheever* strain. Sanders(1) had originally reported that EK virus caused a fatal encephalitis in intracerebrally inoculated mice and rabbits but that rats and guinea pigs were refractory. In 3 of these instances the findings were easily confirmed and in addition the discovery was made that guinea pigs were now also susceptible. The results of intracerebral inoculation follow. Rabbits inoculated with EK virus (0.1 cc) developed fever, and central nervous system (CNS) signs. Death ensued between the seventh and fourteenth days. Rabbits inoculated with two laboratory adapted strains of SLE virus remained well. Mice, following inoculation with EK virus (0.03 cc) began as early as 24 hours to show CNS signs which included rotating along the horizontal axis.

TABLE V. Comparison Between Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses in Intracerebrally Inoculated Animals.

Animal	Epidemic Keratoconjunctivitis	St. Louis Encephalitis
Mice		
Titer	Ca 10^{-8}	Ca 10^{-8}
Incubation (10% susp.)	72 hr	72 hr
CNS signs first appear	24-48 hr	72 hr
Type of CNS signs	Usual and rotating	Usual
Rats		
Young adult	Refractory	Refractory
G. pigs		
Pyrexia	Present	Present
CNS signs	”	None
Mortality	60-70%	”
Rabbits	Fever, CNS, death	No effect

At 48 hours practically 100% of the mice were showing marked symptoms and at 72 hours the vast majority were moribund. Following inoculation with SLE the mice remained well for the first 48 hours but at 72 hours they were all showing marked CNS signs. Guinea pigs injected with EK (0.1 cc) developed fever and weight loss. About 60 to 70% of the animals developed CNS signs and died between the seventh and twelfth days. Guinea pigs inoculated with SLE virus developed fever and some weight loss but all survived the infection. Albino rats were refractory to both EK and SLE viruses. Thus the apparent pathogenicity of the EK virus for rabbits and to a lesser degree for guinea pigs is at present the only method whereby EK virus can be distinguished from SLE. A comparison between results obtained with the two viruses is given in Table V.

Discussion. As a result of the studies reported here it soon became apparent that it was impossible to differentiate the EK and SLE viruses by means of the neutralization test. Nevertheless by other means certain differences were found. The EK virus caused a fatal encephalitis in rabbits and in a majority of the guinea pigs whereas the SLE viruses were ineffective in rabbits and only caused a febrile response in guinea pigs. In mice, intracerebral inoculation of EK virus induced marked CNS signs, which included rotating along the horizontal axis, as early as 24 hours. These signs continued until death occurred at about 72 hours. Intracerebral inoculation of SLE produced signs at about 72 hours but there was no ‘rotating syndrome’. The marked similarities included the crossing over in the neutralization tests and practically identical LD_{50} intracerebral titers in mice. Preliminary tests already indicate that cross-immunity tests in animals do not serve to distinguish the two viruses too well although some differences have been noted. Much of this work has been confirmed by Cheever(4). Further work is in progress and it will be reported later.

What remains unanswered at the present time is the question of whether this is the EK virus or a strain of SLE which can cause disease in rabbits and guinea pigs in addition to the susceptible mouse. Unfortunately no specific immune sera were available to help. If the virus is EK then it is immunologically related to SLE virus and it has now acquired the ability to cause disease in guinea pigs and results in much higher titers in mice than was originally reported. If it is SLE virus it is then unique in two respects. First, SLE does not cause encephalitis in rabbits and second, except for the report by Sulkin, *et al.* (7) in which they found it impossible to repeat their own results, presumably because of different strains of guinea pigs, it is generally agreed that SLE does not cause encephalitis in guinea pigs. Further work along this line is indicated.

Previous reports have tended to show that there is significant crossing over in the in-

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7. Sulkin, S. E., Harford, C. G., and Bronfenbrenner, J., Proc. Soc. Exp. Biol. and Med., 1939, v41, 329.

tracerebral neutralization tests with the sera from animals immune to SLE, Jap B and West Nile viruses(8-10). These results were readily confirmed here but it was surprising to find the high neutralization indexes obtained with the heterologous viruses especially when hyperimmune sera were used. Indexes of one thousand or more were quite frequent and several were rcorded of ten thousand or better. Evidently repeated inoculations of animals with living virus serve not only to raise the level of antibody against the homologous virus but also to bring the level of antibody against the heterologous viruses to a significantly high level. Thus as suggested by Lennette and Koprowski(11) it may be of importance in the future to use sera from convalescent animals rather than hyperimmune animals for identification purposes.

Summary. It is impossible at present to distinguish epidemic keratoconjunctivitis virus from St. Louis encephalitis virus by means of the intracerebral neutralization test in mice. The sera from convalescent epidemic keratoconjunctivitis animals and from convalescent or hyperimmune St. Louis encephalitis animals neutralized epidemic keratoconjunctivitis and St. Louis encephalitis viruses to an equal extent. No differences could be elicited by performing neutralization tests with the keratoconjunctivitis virus that had been through 3 successive rabbit passages. Neutralization tests in which serum dilutions were used against a constant amount of virus also failed to show any differences between the 2 viruses. Considerable cross-neutralization was obtained with the related Japanese B and West Nile viruses. Infectivity tests in animals revealed that rabbits and guinea pigs were susceptible to intracerebral inoculation with keratoconjunctivitis virus but not to St. Louis virus.

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9. Smithburn, K. C. and Jacobs, H. R., *J. Immunol.*, 1942, v44, 9.

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A Possible Relationship Between Viruses of St. Louis Encephalitis and Epidemic Keratoconjunctivitis. (18699)

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The virus of epidemic keratoconjunctivitis first isolated by Sanders(1,2) has recently been shown by Ruchman to be closely related to the virus of St. Louis encephalitis(3). The present communication confirms this fact and, in addition, presents certain data bearing on the relationship of this agent or agents to the clinical disease of epidemic keratoconjunctivitis.

Materials and methods. Two strains of epidemic keratoconjunctivitis virus were examined. The first, EK-TC 17, was obtained from Dr. A. E. Braley in 1947. This was the strain originally isolated by Sanders(1,2) and carried through an undetermined number of mouse and egg passages. During the Second World War it was sent to the Upjohn Co. in Kalamazoo, Mich., where it was carried through another series of mouse and egg passages(4). At the close of hostilities it was returned to Dr. Braley, who confirmed its specific identification by serological and filtration studies(5). During this time the titer

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1. Sanders, M., *Arch. Ophthalmol.*, 1942, v28, 581.

2. Sanders, M., and Alexander, R. C., *J. Exp. Med.*, 1943, v77, 71.

3. Ruchman, I., in press.

4. Calkins, H. E., and Bond, G. C., PROC. SOC. EXP. BIOL. AND MED., 1944, v56, 46.

5. Braley, A. E., personal communication.

of the virus varied from 10^{-5} to 10^{-7}. This strain was subsequently used in protection tests employing gamma globulin *in vitro*(6) and *in vivo*(7); the titer of the virus at this time varied from 10^{-5} to 10^{-6}. The second strain, EK 103-P61, was obtained from Dr. Isaac Ruchman, in July 1950, who, in turn, had received it from Dr. Braley a month previously. While details of the early history of this strain were not available, it had apparently undergone more than 100 mouse passages and was derived from the same pool of material made up by Dr. Murray Sanders in New York City in 1942 as was strain EK-TC 17(3). Attempts to obtain other strains of the virus of epidemic keratoconjunctivitis failed. We were particularly anxious to secure the strain isolated by Maumenee, Hayes, and Hartman(8) which was reported to be antigenically related to *Herpes febrilis* virus. Apparently this strain is no longer available(9). Two strains of St. Louis encephalitis virus, Webster(10) and Hubbard(11), were secured from Dr. W. McD. Hammon. Pools of each of these viral agents were made up in the form of 20% suspensions of infected mouse brain, using 1% bovine albumen solution in broth as the suspending fluid. These were stored in sealed 1 ml ampoules in the CO_2 cabinet until used. All virus preparations were cultured aerobically and anaerobically to insure bacteriological sterility. Rabbits and guinea pigs of both sexes were obtained from a local dealer. Three-four-week-old mice of the Carworth Farm CFW strain were employed in the neutralization and cross immunity tests as well as for the preparation and titration of virus pools. Fertile White Leghorn eggs were used for chick embryo passage experiments.

Titrations of individual virus suspensions were carried out by making serial 10-fold dilutions in 0.2% bovine albumen solution in broth. Five or 6 mice were inoculated with each dilution. The method of Reed and Muench(12) was used in the calculation of LD_{50} titers. Neutralization tests were carried out and the resultant neutralization indices calculated by the methods outlined by Hammon(13), employing undiluted serum and varying amounts of virus. In these tests the virus suspensions were prepared employing 0.4% bovine albumen as diluent, so that when equal amounts of virus suspension and undiluted serum were mixed the final concentration of bovine albumen was 0.2%. These mixtures were placed in a 37°C waterbath for 2 hours; 0.03 ml of each mixture was inoculated intracerebrally into each of 5 or 6 mice. Inactivated normal horse serum was used as a control.

Results. The EK-TC 17 strain has been carried through 4 passages and the EK 103-P61 strain through 2 passages. The LD_{50} titers of these passages have varied from $10^{-7.3}$ to $10^{-8.2}$.

Neutralization tests were carried out with both virus strains employing immune rabbit serum prepared against each of the following encephalitis viruses: St. Louis, Japanese B, and Western Equine. These sera were furnished by Dr. W. McD. Hammon. As shown in Tables I and II, the St. Louis immune serum showed significant neutralization indices against both strains. Slight but not significant protection against one strain was afforded by the Japanese B immune serum, while the Western Equine encephalomyelitis immune serum exerted no demonstrable protective effect. Since these results presented presumptive evidence that the two strains of epidemic keratoconjunctivitis virus were identical, further work was limited to one strain—EK-TC 17.

Host range. Three guinea pigs inoculated intracerebrally with EK-TC 17 developed fever of 3-4 days duration commencing on the

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6. Heyl, J. T., Allen, H. F., and Cheever, F. S., *J. Immunol.*, 1948, v60, 37.

7. Cheever, F. S., and Daikos, G., *J. Immunol.*, 1950, v65, 135.

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9. Maumenee, A. E., personal communication.

10. España, C., and Hammon, W. McD., *J. Immunol.*, 1948, v59, 1.

11. American Type Culture Collection: *Virus and Rickettsial Registry*, 1950, 6.

12. Reed, J. L., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

13. Hammon, W. McD. in *Diagnostic Procedures for Virus and Rickettsial Diseases*, New York, American Public Health Association, 1948, 200.

TABLE I. Epidemic Keratoconjunctivitis Virus Neutralization Tests.

	Strain EK-TC 17: Final virus dilutions								
Serum	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE* immune	5/5†	4/5	1/5	0/5	0/5	—	—	$10^{-4.5}$	500
Jap B‡ ”	5/5	5/5	5/5	2/5	0/5	—	—	$10^{-5.8}$	25
WEE§ ”	—	—	5/5	5/5	5/5	2/5	0/5	$10^{-7.8}$	<10
Normal horse	—	—	5/5	5/5	2/5	2/5	0/5	$10^{-7.2}$	—

* St. Louis encephalitis.

† Numerator: No. of deaths.
Denominator: ” ” mice inoculated.

‡ Japanese B encephalitis.

§ Western equine ”

TABLE II.

	Strain EK 103-P61: Final virus dilutions								
Serum	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE immune	5/5	4/5	0/5	0/5	0/5	0/5	0/5	$10^{-4.4}$	1000
Jap B ”	5/5	5/5	5/5	4/5	1/5	0/5	0/5	$10^{-6.5}$	<10
WEE ”	—	5/5	5/5	5/5	5/5	0/5	0/5	$10^{-7.5}$	<10
Normal horse	—	5/5	5/5	4/5	4/5	1/5	0/5	$10^{-7.4}$	—

fourth day after inoculation. One pig died on the 11th post-inoculation day with no definite neurological signs. The brain was bacteriologically sterile and attempts to isolate the virus by mouse inoculation were successful. Five guinea pigs inoculated with the 2 strains of St. Louis encephalitis virus showed a similar but less well marked febrile response and all recovered. Reinoculation experiments demonstrated cross immunity between the 2 viruses —*i.e.*, St. Louis encephalitis-recovered guinea pigs showed no febrile response after inoculation with epidemic keratoconjunctivitis virus 4 weeks later, and vice versa.

Of 2 rabbits inoculated intracerebrally with EK-TC 17 one remained apparently well throughout the period of observation. The second developed signs suggesting CNS involvement on the 11th post-inoculation day and was sacrificed when moribund on the 14th day. A 20% suspension of brain tissue was prepared and cultured under aerobic and anaerobic conditions; no growth occurred. A few of the mice injected intracerebrally with this material died 96 hours after inoculation, but attempts to pass the agent further failed.

Three groups of 3-4-week-old mice were inoculated intraperitoneally with epidemic keratoconjunctivitis virus (EK-TC 17 strain) and the 2 strains (Webster and Hubbard) of St. Louis encephalitis virus respectively. The intracerebral titers of the 3 virus pools were approximately equal. Each mouse received 0.1 ml of a 10% suspension of virus. The mortality ratios varied considerably; the actual ratios were SLE Hubbard: 4/15 (27%), SLE Webster: 12/15 (80%), EK-TC 17: 15/16 (94%).

No difficulty was experienced in propagating the virus of epidemic keratoconjunctivitis (strain EK-TC 17) in 8-day embryonated hens' eggs. The yolk sac route of inoculation was employed. As a general rule, the embryos began to die in significant numbers between 48 and 72 hours after inoculation. When sacrificed at this time the embryos usually appeared hemorrhagic. After removal of the eyes, beak and feet the remainder of the embryo was ground up in 1% bovine albumen solution so as to give a 10 or 20% suspension, and this was used as inoculum for the next passage. The LD_{50} titers of the first 10 passages varied from $10^{-4.5}$ to $10^{-7.7}$; the titers obtained with the 4th and subsequent passage materials were somewhat higher than has been the result when St. Louis encephalitis virus has been inoculated by the same route.

Mice immunized against St. Louis encephalitis virus (Hubbard strain) by a series of 3 graded intraperitoneal injections at weekly intervals showed marked immunity to intracerebral challenge with the homologous virus 10 days after the last immunizing injection as compared to control animals of the same age.

TABLE III. Cross Immunity Tests. Mice Immunized Against SLE Hubbard.

Mice	Challenge virus	Virus dilutions									LD_{50}	NI
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
SLE immune	SLE	0/7	1/8	1/7	0/7	1/7	0/8	1/8	0/5	—	$<10^{-1}$	>2000000
Controls	SLE	—	—	—	8/8	9/9	8/8	6/9	1/8	0/8	$10^{-7.3}$	—
SLE immune	EK	9/10	9/9	9/9	10/10	10/10	6/9	2/10	1/10	—	$10^{-6.5}$	20
Controls	EK	—	—	—	7/7	7/7	7/7	6/7	3/7	0/7	$10^{-7.8}$	—

TABLE IV. Cross Immunity Tests. Mice Immunized Against EK-TC 17.*

Mice	Challenge virus	Virus dilutions									LD_{50}	NI
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
EK immune	EK	10/10	10/11	11/11	9/9	8/9	6/10	2/7	0/10	—	$10^{-6.2}$	16
Controls	EK	—	—	—	9/9	8/8	8/8	5/7	1/8	0/7	$10^{-7.4}$	—
EK immune	SLE	1/10	2/8	2/9	3/9	3/9	3/9	0/8	0/7	—	$10^{-1.7}$	790000
Controls	SLE	—	—	—	8/8	8/8	8/8	7/8	2/8	0/7	$10^{-7.6}$	—

* Exp. 3.

When challenged with epidemic keratoconjunctivitis virus only a slight degree of increased immunity was demonstrated (Table III). Mice were immunized against epidemic keratoconjunctivitis virus (strain EK-TC 17) in similar fashion and subsequently challenged with the homologous and the heterologous viruses. In 3 different experiments only a minimal amount of acquired immunity against the homologous virus (EK) was demonstrated. In all 3 experiments the degree of acquired immunity against the heterologous virus (St. Louis Hubbard) was greater, although the actual neutralization indices showed considerable variation. The explanation of these findings is not clear (Table IV).

Attempts were made to secure specific immune sera against epidemic keratoconjunctivitis virus from outside sources. Through the kindness of Dr. Murray Sanders, serum drawn from a patient 8 years after an experimental infection with EK virus was made available to us. During the course of his experimental but clinically typical infection, this patient (M.S.) developed neutralizing antibodies against the specific virus(2). To his knowledge he had never had any contact with St. Louis encephalitis virus. Two convalescent serum specimens from the Schenectady outbreak of 1943(14) were furnished us through the kindness of Dr. R. F. Korns. One was a sample from a pool of convalescent serum obtained in 1943 from patients, all of whom had suffered clinically typical attacks of epidemic keratoconjunctivitis. An undetermined number of these individuals had been hyperimmunized with epidemic keratoconjunctivitis virus vaccine during convalescence (but prior to bleeding) as well. The second specimen was drawn in 1943 from an individual patient (Crouch) who during convalescence from a typical attack of the disease had received several injections of the same vaccine prior to being bled as a source of hyperimmune serum. Neutralization tests were set up with these specimens against the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis.

As seen in Tables V and VI, patient MS possessed moderate levels of protective antibody against both viruses. Both patient Crouch and the pool of convalescent sera from the Schenectady outbreak showed significant levels of protective antibody against St. Louis encephalitis virus (Table V). Neither specimen showed any protective effect against the epidemic keratoconjunctivitis virus (Table VI).

Discussion. The findings reported here confirm Ruchman's observations(3) that a close serological relationship exists between the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis. Further and more extensive tests must be carried out before it can be determined if the differences noted are more than one would expect to exist between recog-

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14. Korns, R. F., Sanders, M., and Alexander, R. C., *Am. J. Public Health*, 1944, v34, 567.

TABLE V. St. Louis Encephalitis Virus. Neutralization tests.

	SLE Hubbard strain: Final virus dilutions									
Serum	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE immune	—	5/5	1/5	0/4	0/5	0/5	0/5	—	$10^{-3.6}$	7940
Patient M.S.	—	5/5	5/5	2/5	1/5	1/5	0/5	0/5	$10^{-5.2}$	200
Normal horse	—	—	5/5	5/5	5/5	4/5	1/5	0/5	$10^{-7.5}$	—
SLE immune	6/6	5/5	2/6	0/6	0/6	0/6	—	—	$10^{-3.7}$	5010
Schen. Conv. pool	6/6	6/6	5/6	0/6	0/5	0/5	0/6	0/6	$10^{-4.4}$	1000
Patient Crouch	6/6	6/6	6/6	4/6	1/6	0/5	0/6	0/6	$10^{-5.4}$	100
Normal horse	—	6/6	6/6	6/6	5/6	5/6	1/6	0/6	$10^{-7.4}$	—

TABLE VI. Epidemic Keratoconjunctivitis Virus. Neutralization tests.

	Strain EK-TC 17: Final virus dilutions									
Serum	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE immune	5/5	4/5	3/5	1/5	0/5	0/4	—	—	$10^{-4.2}$	3160
Jap B	—	5/5	5/5	5/5	2/5	0/5	0/5	—	$10^{-5.8}$	79
Patient M.S.	—	5/5	5/5	3/5	0/5	0/5	—	—	$10^{-5.2}$	316
Normal horse	—	—	5/5	5/5	5/5	4/5	2/5	0/5	$10^{-7.7}$	—
SLE immune	6/6	4/6	2/6	1/6	0/6	0/6	—	—	$10^{-3.5}$	1590
Schen. Conv. pool	6/6	5/5	6/6	6/6	3/5	2/6	0/5	0/6	$10^{-6.5}$	<10
Patient Crouch	6/6	6/6	6/6	6/6	5/6	4/6	0/6	0/6	$10^{-7.1}$	<10
Normal horse	—	6/6	6/6	6/6	6/6	2/6	0/6	0/6	$10^{-6.7}$	—

nized strains of St. Louis encephalitis virus (*e.g.* Webster and Hubbard). The serological results presented suggest that patient MS did suffer from an infection of an agent closely related to the virus of St. Louis encephalitis, if it can be assumed that no other unrecognized exposure to the latter agent had occurred. The results obtained from the Schenectady outbreak are more difficult to interpret since artificial immunization as well as natural disease presumably played a part in the stimulation of antibody production, but it seems probable that an agent closely related to St. Louis encephalitis virus was the causative agent of the outbreak. The failure to demonstrate neutralizing antibodies against the virus of epidemic keratoconjunctivitis is difficult to explain in the face of the strong presumptive evidence presented by Korns, Sanders, and Alexander(14) that unvaccinated individuals involved in the same outbreak developed specific antibodies against this agent in the course of typical attacks of the disease. It is obvious that outbreaks of epidemic keratoconjunctivitis as they occur today should be carefully studied and that in addition to carrying out the conventional antibody studies every effort should be made to isolate and identify the etiological agent. From the epidemiological point of view it is interesting that a good proportion of the reported outbreaks of epidemic keratoconjunctivitis have occurred under conditions which did not favor arthropod transmission of the agent.

As first pointed out by Ruchman(3) both strains of epidemic keratoconjunctivitis virus have shown a marked increase in virulence as measured by intracerebral titrations in mice. The explanation for this finding is not clear. This agent (strain EK-TC 17) was titrated simultaneously in Carworth Farm CFW mice and in the Tumblebrook strain of Swiss white mice. This latter strain had been employed in the studies involving the protective effect of gamma globulin(6,7). The LD_{50} titer of the virus pool was identical in both titrations. Similar results were obtained when the Hubbard strain of St. Louis encephalitis virus was titrated simultaneously in the two groups of animals. It seems unlikely, therefore, that the strain of mouse employed was responsible for this finding.

Summary. Evidence has been presented which confirms Ruchman's observations that the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis are closely related. The importance of careful study of current outbreaks of epidemic keratoconjunctivitis as a means of assessing the significance of these findings is stressed.

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Ntaya Virus. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda.* (18700)

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The purpose of this communication is to record the isolation in 1943 of a previously unknown neutrotropic virus which is now, along with other recently discovered viruses, being studied further in New York. The circumstances concerned with its isolation, together with subsequent observations, render it impossible to state its exact origin but it is believed that it was derived from wild mosquitoes. The name applied is in respect to the locality where the mosquitoes were caught, the Ntaya swamp in Bwamba County, Uganda Protectorate, British East Africa.

Ntaya virus was encountered during the course of coordinated laboratory and field investigations, the purpose of which was to discover the insects responsible for the transmission of yellow fever in extra-human cycles of natural infection. Yellow fever is enzootic among the wild primates of Bwamba County (Haddow, *et al.*)(1), and is also either endemic or repetitively epidemic among the human population of the area (Mahaffy, *et al.*)(2). The procedure in the attempts to discover the vector (or vectors) was to capture mosquitoes in selected localities, to classify them, and then to inoculate suspensions of them into susceptible laboratory animals (mice and rhesus monkeys) to test for the presence of virus. The methods which are effective for the isolation of yellow fever virus (Mahaffy, *et al.*)(2); Smithburn and Haddow(3); Smithburn, Haddow, and Lumsden(4), have also yielded several other viruses, 2 of which were previously known: Rift Valley fever (Smithburn, Haddow, and Gillett)(5), and Mengo (Dick, Smithburn, and Haddow)(6), the latter being identical with encephalomyocarditis, M. M. and Columbia S-K viruses (Dick)(7); Warren, Smadel, and Russ(8). Five other agents, each apparently hitherto unknown, were isolated in the same manner, including: Semliki Forest (Smithburn and Haddow)(9), Bunyamwera (Smithburn, Haddow, and Mahaffy)(10), Uganda S(11) and Zika(12) viruses, in addition to the Ntaya virus discussed here. The details of methods employed have been given in previously mentioned papers.

As has been stated elsewhere (Haddow) (13), the Bwamba lowlands include a large tract of uninhabited primeval forest and a densely populated agricultural area. The latter is much cut up by deep valleys which contain relict belts and patches of primary forest, some of them continuous with the main Semliki Forest. These relict strips have survived mainly in localities unsuited to agricultural development. The Ntaya swamp is a marshy reach of the Manjuguja River, near its source, lying in a deep valley about 1½ miles southwest of Bundibugyo. The relict forest there in 1943 was very dense and tangled, its most characteristic feature being a predominance of old oil palms (*Elaeis*). It was very small in extent—not more than half a square mile in all—and had no connection with the main uninhabited forest, being surrounded on all sides by native plantations and dwellings. This forest has now almost entirely disappeared.

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* The studies reported here were made in part at the Yellow Fever Research Institute, Entebbe, Uganda (presently the Virus Research Institute), then supported jointly by the Medical Department of the Government of Uganda Protectorate and the International Health Division of The Rockefeller Foundation, and in part in the laboratories of the International Health Division in New York.

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In the early months of 1943 Bwamba County was suffering an unusual period of drought, and the Ntaya swamp area was the only locality in which it was possible to collect mosquitoes in sufficient numbers to warrant a full-time catch. Moreover, the catch which was made yielded so few mosquitoes of species which were interesting or suspect as possible vectors of yellow fever that the usual procedure of inoculating highly suspect species separately was dispensed with. Instead, only the 120 *Eretmapodites* spp. and the 66 *Aedimorphus* spp. were treated separately. No pathogenic agent was recovered from these insects. All of the other mosquitoes taken in 5 successive days of catching were grouped for the inoculations. The latter lot, which presumably gave origin to the Ntaya virus, included:

Taeniorhynchus (Coquillettidia) pseudoconopas Theo.	4
Uranotaenia alboabdominalis Theo.	4
Theobaldia (Theomyia) fraseri Edw.	6
T. (C.) aurites Theo.	2
Aedes (Stegomyia) simpsoni Theo.†	1
A. (S.) apicoargenteus Theo.	12
A. (S.) africanus Theo.‡	3
A. (Dunnius) albomarginatus Newst.	2
Culex (Lutzia) tigripes group Theo.	31
C. (Culex) poicilipes Theo.	4
C. (C.) pruina Theo.	104
C. (C.) moucheti Ev.	271
Culex Ev. spp. indet. (about 12 species)	874
Total	1318

These 1318 mosquitoes were dispatched from the field station in Bwamba to the central laboratory at Entebbe. On arrival Feb. 22, 1943, all the living mosquitoes were chloroformed and ground up, together with 162 which had died en route, in 10% normal monkey serum in physiological saline. A portion of the supernate of the suspension was Seitz-filtered and inoculated intracerebrally into mice and the remaining unfiltered supernate was injected subcutaneously into normal rhesus monkey No. 276.

The monkey exhibited neither fever nor other signs of illness following inoculation. Of the 6 mice inoculated with the mosquito suspension 2 exhibited paralysis, 1 on the 7th and 1 on the 9th day, while the other 4 remained well. Brain passages were made from the 2 mice showing paralysis, both unfiltered and Seitz-filtered suspensions being used, with resulting successful transmission in each instance. One to 3 of 12 mice survived in each of the first 3 intracerebral passages, and there were occasional survivors through 11 passages, after which all the mice in the passage series died. Duplicate lines were successfully transmitted through 100 successive brain-to-brain passages in mice. Owing, doubtless, to adaptation to the brain of the white mouse as a host, there was gradual diminution of the average survival time of mice in the serial transfers. The average survival time of the 74 mice which succumbed in the 2d to 11th passages, disregarding 20 which survived, was 8.1 days; the average survival time among 101 mice of the 91st to 100th passages, in which there were no survivors, was 5.9 days. Seitz filtrates of brain were occasionally used for passage, invariably with success, and with no noteworthy prolongation of survival time. The agent was also found to be filterable through all grades of Berkefeld filters.

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† The single specimen of *Aedes simpsoni* was originally determined as *A. metallicus* Edw. At a later date comparative material became available and it was found that what had been taken for this species was a rare variant of *A. simpsoni* with an aberrant scutal pattern (Haddow, *et al.*) (14). Had it been realized that this mosquito was a specimen of *A. simpsoni*, it would not have been included in the mixed batch, as this species was already known to be an important vector of yellow fever among human beings in Bwamba County.

‡ At this time *Aedes africanus* was not particularly suspected of being a forest vector of yellow fever, as it eventually proved to be (Smithburn, Haddow, and Lumsden) (4); had it been, these specimens would have been inoculated separately.

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Rhesus No. 276, which received the mosquito suspension, exhibited no neutralizing antibody in its serum either 3 weeks or 8 months following the inoculation of the mosquito suspension. From this evidence one might suspect that the virus did not derive from the mosquitoes. However, 2 subinoculations of 10% mouse brain from the paralyzed mice of the first passage were made to another normal rhesus, No. 282, and this animal likewise failed to develop antibody as a result. The latter was found to be capable of producing antibody when it developed humoral immunity following 2 further inoculations of concentrated mouse brain passage virus. It appeared, therefore, that the Ntaya virus is neither highly pathogenic nor strongly antigenic for rhesus monkeys, in view of which the failure to demonstrate antibody in rhesus No. 276 does not preclude the derivation of the virus from the mosquitoes. No agent similar to Ntaya virus was ever before or since encountered in the stock mice at Entebbe. It is therefore our opinion that the virus was, in fact, derived from the Ntaya mosquitoes, although proof of this point is impossible to obtain.

Properties of Ntaya virus. Comprehensive studies of this agent have not yet been made, owing in part to uncertainty as to the origin of the virus up to the time that monkey No. 282 finally developed neutralizing antibody. The data available concerning it are presented as a preliminary study and further information will be forthcoming when studies now in progress are completed on this and other viruses recently isolated in the yellow fever laboratories in East and West Africa, Colombia and Brazil.

Pathogenic properties in mice. After an incubation period of 3 to 7 days, depending on the passage level and the dilution of the inoculum, mice inoculated intracerebrally with mouse brain Ntaya virus show the first signs of illness. Many of the mice show paralysis, first of the hind quarters, and later of the fore, while these and others showing no paralysis exhibit a roughening of the pelage, mild tremors, and occasionally convulsions. About 48 hours usually elapse between the onset of symptoms and death, prior to which the majority of mice are either paralyzed or prostrate. Deaths occur between the 5th and 14th days, depending on the passage level and the dose of virus inoculated. Intranasal and intraperitoneal inoculation of the virus into mice did not cause illness, the intraperitoneal inoculations proving harmless even after intracerebral inoculation of an irritant, sterile starch solution, as practiced in the intraperitoneal yellow fever protection test (Sawyer and Lloyd(15). In the earliest passages the titers of virus in the brains of inoculated mice were only 10^{-4} to 10^{-5}. With continued passage there was a moderate increase in potency, titers at the 56th passage reaching 10^{-6} to $10^{-6.5}$. Titers as high as 10^{-7} have not been observed.

Pathogenicity for rhesus monkeys and hamsters. Other than the aforementioned two monkeys, only one additional rhesus has been inoculated with Ntaya virus. Rhesus M4898 was inoculated subcutaneously in New York with 8400 LD_{50} of virus, failed to show either fever or other signs of illness, and, like monkeys Nos. 276 and 282 in Uganda, developed little or no neutralizing antibody. Rhesus M4898 received a further 3 injections of concentrated virus, to which there was no clinical response, yet the animal, like rhesus No. 282, then exhibited potent neutralizing antibody in its serum. Young Syrian hamsters inoculated intracerebrally or subcutaneously exhibited no clinical reactions to the virus. One hamster inoculated intracerebrally with 205 LD_{50} developed little or no antibody as a result of the inoculation; another inoculated subcutaneously with 6800 LD_{50} developed antibody in moderate titer. The latter may indicate that hamsters are more susceptible to the virus than rhesus monkeys. The pathogenicity of the virus for other species of animals has not been tested.

Preservation of the agent. Ntaya virus

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15. Sawyer, W. A. and Lloyd, W., *J. Exp. Med.*, 1931, v54, 533.

TABLE I.
Intracerebral Neutralization Test in Mice Showing the Development of Antibody in Rhesus No. 282 after 4, but not after 2, Injections of Concentrated Virus Suspension.

Serum	No. injections of virus	Interval after last inj., days	Titer of virus, log	LD_{50} neutralized, log
Normal rhesus pool	0	—	6.3	—
No. 282 Pre-inoculation	0	—	6.6	—
” ” Mar. 15	2	12	6.15	0.45
” ” May 18	4	12	3.4	3.2
” ” Oct. 25	4	172	3.5	3.1

may readily be preserved by lyophilization. Eighth passage virus was titrated before and 20 days after desiccation, with the following results:

Titer of original suspension $10^{-6.31}$
Titer 20 days after desiccation $10^{-5.94}$

The aforementioned desiccated virus was still potent 3½ years later, after shipment without refrigeration to New York. A desiccated lot of 13th passage virus prepared in New York showed no significant decline in potency within 15 months. The virus may also be preserved, at least for short periods, by storage of the mouse brain suspension in the frozen state, either at –25°C in a mechanical refrigerator, or in the solid CO_2 cabinet.

Immunological reactions. When a suitably potent immune serum was finally obtained, it was found possible to perform intracerebral neutralization tests in mice essentially according to the method developed by Theiler(16) for the intracerebral yellow fever neutralization test. 0.3 cc portions of suitable dilutions of fresh mouse passage or desiccated virus are added to 0.3 cc portions of whole sera, the mixtures are incubated 2 hours at 37°C and inoculated intracerebrally in 0.03 cc quantities into susceptible Swiss mice. Results of the test showing the development of antibody in rhesus No. 282 are shown in Table I.

It is noteworthy that the immunity acquired by rhesus No. 282 was retained from May, 1943, without any further known contact with Ntaya virus, and serum taken from the animal on September 19, 1949, still contained abundant antibody. Thus, although a potent immune response is not easily evoked, once the antibody develops it is well retained.

Immediately after its isolation Ntaya virus was tested against yellow fever immune serum, with negative results. Recently, reciprocal cross neutralization tests were done with Ntaya virus and a number of other neurotropic viruses without any strong cross reaction being observed. The agents tested in the latter studies included: Bwamba fever, West Nile, Semliki Forest, Bunyamwera, Mengo, Uganda S and Zika viruses, all isolated in Uganda; Kumba virus, isolated in the Cameroons; Anopheles A, Anopheles B, and Wyeomyia viruses, isolated in Colombia (Roca Garcia)(17); Ilhéus virus, isolated in Brazil (Laemmert and Hughes)(18); and 10 previously known agents—Western, Eastern and Venezuelan equine encephalomyelitis, St. Louis, Japanese B and Russian spring-summer encephalitis, louping ill, encephalomyocarditis, Dengue (Hawaiian strain) and yellow fever viruses.

In an effort to ascertain whether or not Ntaya virus attacks man, the sera of 43 persons residing near the Ntaya swamp were tested for antibody against the virus. None of these exhibited any neutralizing power. Further studies of this nature are anticipated but have not yet been made. At this time, therefore, its natural hosts remain unknown.

Summary. A filterable neurotropic virus was isolated from mice inoculated intracerebrally with a mixed lot of mosquitoes captured in western Uganda. The agent is believed to be hitherto unknown and has been named Ntaya virus in respect of the locality in which the mosquitoes were caught. Certain of its pathogenic and immunological properties are discussed.

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Resistance of Red Cells to Dialuric Acid of Premature Infants with Retrolental Fibroplasia.* (18701)

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The etiology of retrolental fibroplasia in premature infants is not known. It has been suggested, however, that an increased incidence of the disease occurred following (a) oxygen therapy, (b) the administration of iron, (c) the administration of water-miscible vitamin preparations(1), and (d) the use of certain detergents(2). Owens and Owens(3) reported that a deficiency of vit. E might be the primary factor in the incidence of retrolental fibroplasia. Callison and Orent-Keiles(4) noted abnormalities of the eye occurring in young vit. E-deficient rats.

The experiments of Rose and György(5) furnish a sensitive method for the detection of the initial stages of vit. E deficiency in the rat. *In vitro* the erythrocytes from deficient rats were readily hemolyzed by compounds such as dialuric acid and alloxantin, and this hemolysis was prevented by the addition of alpha-tocopherol either to the diet or *in vitro*. Using this procedure, we have investigated the possibility that retrolental fibroplasia in premature infants may be correlated with a deficiency of tocopherol.

Experimental. Premature infants admitted to Colorado General Hospital during the latter half of 1950 were studied. All infants were fed partially skimmed cow's milk-carbohydrate mixtures with added vit. A and D. None received any added vit. E in the diet. Blood samples were obtained by heel pricks from 5 normal premature infants and from 9 infants showing varying degrees of retrolental fibroplasia. The erythrocytes were tested for sensitivity to hemolysis by dialuric acid employing the procedure of Rose and György(5). For the hemolysis test 10 drops of blood were collected in 2 ml of a solution containing 0.9% of sodium chloride and 1% of sodium citrate. The blood was centrifuged, the supernatant fluid removed and 0.9% sodium chloride added to make a 5% suspension of the blood cells. To 0.25 ml of this suspension in a small tube were aded 0.18 ml of phosphate buffer and 0.07 ml of a 0.1% solution of dialuric acid in the buffer. The tubes were incubated for 15 minutes at 37°C, after which they were kept at room temperature and observed for at least 4 hours. A control tube without dialuric acid was run with the test sample. The validity of the procedure for the detection of a deficiency of vit. E in rats was confirmed. One group of 4 rats was fed a diet free from vit. E (General Biochemicals); a second group of 4 animals was fed a stock commercial diet. In 46 days the red blood cells obtained from the deficient animals were almost completely hemolyzed by dialuric acid, whereas those from the control animals showed no hemolysis. These results are included in Table I.

Results. We have never observed any hemolysis of the erythrocytes of infants by dialuric acid. The red blood cells from normal premature infants and from infants with retrolental fibroplasia showed complete resistance to hemolysis by dialuric acid in the concentrations used (Table I).

Rose and György also studied the litters of rats receiving a stock diet and found that immediately after parturition the erythrocytes of the newborn animals were hemolyzed by dialuric acid. This sensitivity to dialuric acid disappeared within several days.

We have examined 5 samples of cord blood obtained from full term infants. No hemolysis was noted. We have also examined the

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* Assisted by a grant from the Association for the Aid to Crippled Children.

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TABLE I (a). Hemolysis of Red Cells from Vit. E-Deficient Rats by Dialuric Acid.

Exp. No.	Diet	Days after diet started	Hemolysis in 4 hr
1	E-deficient	8	0
		21	0
		46	complete
2	Purina Lab. Chow	46	0

Four animals were used in each experimental group.

TABLE I (b). Resistance of Red Cells to Dialuric Acid of Premature Infants with Retrolental Fibroplasia. No hemolysis in 4 hr.

Subject	Birth wt, g	Age when tested, days	Wt when tested, g	Retrolental fibroplasia
Ma.	880	88	2250	e*
Mi.	1008	84	2310	a
C.	1035	79	2600	a
G.	1148	52	1990	e
G.	1148	75	2370	e
A.	1150	86	2250	a
J.	1268	76	2560	e
L.	1277	64	2400	0
Al.	1305	51	2130	0
F.	1365	28	1460	e
F.	1365	63	2450	r
W.	1417	75	2240	a
Jo.	1470	15	1540	0
R.	1644	57	2860	a
Z.	1750	52	2950	0
Re.	2197	20	2095	0

* e = early; a = advanced; r = regressed.

blood from guinea pig embryos obtained by Caesarian section. No hemolysis was detected in the three litters examined. The guinea pigs had been fed a stock diet of Purina Rabbit Pellets supplemented with lettuce.

Discussion. We have found no correlation between the occurrence of retrolental fibroplasia in premature infants and a deficiency of vit. E as measured by the hemolysis test. It should be mentioned, however, that even if a vit. E deficiency existed in the premature infant, it might not be detected by the procedure employed, which has been validated for the rat only. Mason(6) has summarized the differences in pathology of vit. E deficiency in different species.

Summary. 1. The erythrocytes of 5 normal premature infants and of 9 with retrolental fibroplasia have been examined for a deficiency of vit. E by the hemolysis test of Rose and György. The red blood cells from all of the infants were resistant to hemolysis by dialuric acid. 2. We have confirmed the validity of the hemolysis test for a deficiency of tocopherol in the rat.

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Lethal Infection with Coxsackie Virus of Adult Mice Given Cortisone.

(18702)

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Coxsackie viruses are characterized by virulence for immature mice or hamsters and avirulence for adults of either species. In fact, this unique property is the single biological characteristic which defines this large group of viruses.

Recent studies in this laboratory which demonstrated increased influenza A, B, and mumps virus multiplication in embryonated eggs injected with cortisone(1), and observations of Shwartzman(2) on enhancement by cortisone of poliomyelitis infection in mice and hamsters, suggested the use of this hormone to essay an alteration in the insusceptibility of the adult mouse to Coxsackie viruses.

Materials. Viruses. The Conn. 5 strain of Coxsackie virus (previously obtained through the courtesy of Dr. Joseph L. Melnick) and the RB strain of virus, recovered in this laboratory from the stool of an 8-year-old boy

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suffering from epidemic pleurodynia, were employed in the present investigation. Preliminary cross-neutralization tests have shown no antigenic relationship between the Conn. 5 and RB viruses. *Sera.* Sera were heated at 56°C for 30 minutes before use in neutralization tests. *Cortisone.* Cortisone acetate (Cortone, Merck) was injected subcutaneously in the dosage indicated. *Control materials.* Pfanstiehl peptone broth and 0.85% NaCl solution buffered to pH 7.2 with phosphate were used as control injections. *Mice.* Rockefeller Institute Swiss mice were used in all experiments. Those referred to as "suckling mice" were less than 1 day old, and "adult mice" were sexually mature, weaned animals averaging 16 g in weight and 3-4 weeks in age, unless otherwise specified.

Procedure. One subcutaneous injection of cortisone was administered to mice 1-2 hours prior to the inoculation of virus. Light ether anesthesia was used when mice were injected by the intracerebral route. Mice were observed at least twice daily for signs of illness or death. In all experiments groups of control mice were injected with both cortisone and broth in quantities and by routes identical with those employed with virus-inoculated animals in order to define the incidence of non-specific mortality in mice not given virus. Viral suspensions were administered in amounts approximately proportional to the size of the mice inoculated (0.05 cc virus/1.5 g of mouse) when the intraperitoneal route was used. All viral inocula contained penicillin and streptomycin, and were bacterially sterile on ordinary culture media. Tissues for passage were obtained from moribund or newly dead animals, except in the case of control animals, which were killed while in apparent good health.

Results. Preliminary experiments indicated that most 16-g adult mice survived the subcutaneous administration of 2.5-5.0 mg cortisone for at least 8 days. Accordingly, these dosages in single injections have been employed. In all experiments to date the concomitant administration of cortisone and Coxsackie virus has resulted, in less than 8 days, in the death of all animals so inoculated. A representative experiment with Conn. 5 virus is summarized in Table I. Three days following inoculation all mice given cortisone and virus were found dead, while control mice, given either virus or cortisone singly, were alive after 8 days when the experiment was terminated.

TABLE I.
Lethal Effect of Coxsackie Virus (Conn. 5) in Adult Mice Given Cortisone.

Preliminary injection (s.c.)		Inoculum		
	mg	(i.p.)	D/t†	Day
Cortisone	5.0	Virus*	5/5	3rd (dead)
,,	2.5	,,	5/5	,, ,,
Saline		,,	0/10	8th (killed)
Cortisone	5.0	Broth	0/5	,, ,,
,,	2.5	,,	0/5	,, ,,

* 40,000 LD_{50} (0.2 cc), determined in infant mice.

† D = No. of mice which died; t = total mice in group.

It was important to ascertain: (1) whether viral multiplication had occurred, (2) whether the lethal effect was serially transmissible, and (3) whether viral multiplication and the lethal effect could be neutralized by specific antiviral serum. Data answering each of these questions in the affirmative are presented in Table II. No virus was demonstrated in the brains of mice injected with virus alone. On 2nd passage virus titers in cortisone-injected mice exceeded concentrations possible on the basis of mere carry-over of non-multiplying virus. Both viral multiplication and death were prevented by specific immune serum in mice given virus and cortisone.

Additional experiments to determine the age limit of cortisone-induced susceptibility have established that 35-g mice 17 weeks of age are subject to lethal infection if injected with 7.5 mg of cortisone.

To establish whether infection of the adult mouse was possible with Coxsackie virus not previously passed in infant mice, and derived directly from a human source, the experiments outlined in Table III were carried out. A 10% suspension of stool from which virus (RB) had been previously recovered in 1-day-old mice (but *not* in 10-day-old mice) was inoculated by the intraperitoneal of intracerebral route into adult mice pretreated with cortisone. All mice inoculated by the intra-

TABLE II. Serial Passage and Specific Neutralization of Coxsackie Virus (Conn. 5) in Adult Mice.

Passage No.	Preliminary injection (s.c.)	mg	Inoculum: Virus	Inoculum: Serum	D/t	Titer of adult brain in suckling mice
1	Cortisone	5.0*	Suckling mouse susp. 10^{-2}†	—	5/5	$10^{-3.3}$
2		”	Brain 10^{-1} +	N.S.‡	5/5	$10^{-2.5}$
”		”	” ” +	I.S.§	0/4	0‖
3		”	” ”	—	2/2	—
1	Saline		Suckling mouse susp. 10^{-2}		0/5	0
2			Brain 10^{-1} +	N.S.	0/5	—

* Followed in 48 hr by additional 2.5 mg, in first passage only.

† 6,000 LD_{50} (0.03 cc).

‡ Normal mouse serum.

§ Conn. 5 antiserum (mouse).

‖ Lowest dilution tested, 10^{-2}.

TABLE III. Recovery and Serological Identification in Adult Mice of Coxsackie Virus (RB) from Human Feces.

Passage No.	Prelim. injec. (s.c.)	mg	Inoculum: Virus	Inoculum: Serum	Inoculum: Route	Age of mice, days	D/t	Day	Titer of adult brain in suckling mice
1	—		10^{-1} stool	—	i.p.	1	13/15	8th (dead)	—
”	—		” ”	—	”	10	0/8	” (killed)	—
1	Cortisone	5.0	10^{-1} ”	—	i.p.	21+	6/6	3rd (dead)	$>10^{-3}$
”		”	” ”	—	i.c.	”	6/6	7th ”	$>10^{-3}$
2		”	10^{-2} brain	N.S.*	i.p.	”	5/5	4th ”	$10^{-4.3}$
”		”	” ”	I.S.†	”	”	0/5	8th (killed)	—
3		”	10^{-5} ”	—	”	”	4/4	8th (dead)	—
1	Saline		10^{-1} stool	—	i.p.	21+	2/5	5th, 9th (dead)	(see text)
”			” ”	—	i.c.	”	0/5	9th (killed)	$<10^{-2}$
2			10^{-2} brain	—	i.p.	”	0/5	8th ”	0‡

* Normal mouse serum.

† RB antiserum (mouse).

‡ Lowest dilution tested, 10^{-1}.

peritoneal route were dead in 3 days, while mice receiving virus by the intracerebral route succumbed more slowly, but all had died 7 days after inoculation. It should be noted that animals inoculated intracerebrally received one-tenth the volume of inoculum introduced by the intraperitoneal route. Brains of mice inoculated by either route titered more than 10^{-3} in suckling mice. A 10^{-2} dilution of brain tissue from mice which had been inoculated intraperitoneally with stool promptly produced death in all animals when passed with normal mouse serum, but no evident disease or death in the presence of specific immune serum against the RB virus. No deaths occurred in a group of mice pretreated with saline and inoculated with this brain suspension, reaffirming dependence of viral virulence upon cortisone administration. Titration in suckling mice of pooled brains of mice which died in the second passage disclosed a viral concentration of $10^{4.3}$. A comparable titration of this material in adult mice pretreated with cortisone resulted in death by the 8th day of all mice inoculated with the highest dilution employed (10^{-5}). This latter experiment thus simultaneously demonstrates: (1) the serial transmissibility through 3 passages of Coxsackie virus in cortisone-treated adult mice; and (2) that susceptibility of such adult mice is at least as great as that of infants. When the stool inoculum employed in the preceding experiments was injected into control mice not given cortisone, deaths occurred in none of the animals injected intracerebrally and in only 2 of 5 mice injected by the intraperitoneal route.

In the latter group residual virus was demonstrable in the brain of a healthy-appearing mouse killed 3 days after inoculation, but was not detected in brains of survivors killed on the last day on which death occurred in this group. It is therefore improbable that the 2 deaths in these mice were related to viral multiplication. Passage of the 3rd day brain tissue known to contain virus failed to kill saline-injected mice, and virus was not demonstrated in the brains of animals so inoculated.

In adult mice dying of infection with either Coxsackie virus employed, paralysis, tremor, and convulsions were not perceived. Conjunctivitis was noted occasionally in mice given cortisone alone, but was almost universally present in animals inoculated with virus as well. Two to 3 days following inoculation some animals became briefly hyperexcitable, but in most mice disease was manifested by huddling, ruffled fur, arched backs, labored respirations, progressive unresponsiveness and lethargy which proceeded to stupor and sudden death. Gross examination of abdominal viscera post mortem has disclosed splenic atrophy and hepatic enlargement and congestion. Histologic studies are in progress.

Discussion. The experiments described show that adult Swiss mice may be lethally infected with Coxsackie virus if such mice are administered a single dose of cortisone prior to the inoculation of virus. Furthermore, viral multiplication in the tissues of the adult mouse, as well as serial passage and specific neutralization of virus have been demonstrated in this ordinarily insusceptible host. Although focal muscle lesions have previously been noted in 10-12-g mice, attempts to propagate Coxsackie virus either in series or by alternate passage through suckling and weaned mice have met with failure in the past(3).

It is a matter of great interest that the administration of a chemically defined substance may convert a state of apparent complete insusceptibility to one of marked susceptibility to a viral infection. It will be important to determine the range and specificity of this phenomenon by the study of allied steroids and other viruses. Elucidation of the mechanism of the phenomenon will obviously be dependent upon the acquisition of further knowledge concerning the metabolic action of cortisone in the murine host.

Summary. 1. Adult mice, ordinarily insusceptible to Coxsackie virus infection, may be lethally infected if preliminarily administered cortisone. 2. Multiplication, serial passage, and specific neutralization of Coxsackie virus in adult mice have been demonstrated. 3. Recovery of Coxsackie virus from a human source has been accomplished in adult mice.

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Nature of Multiple Forms of the *Lactobacillus bulgaricus* Factor (LBF).* (18703)

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In previous reports, the discovery(1,2) and purification(2,3) of a growth factor (designated LBF) required by *Lactobacillus bulgaricus* and many other lactic acid bacteria (4,5) was described. Bioautographic studies

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showed that LBF exists in natural materials in several chromatographically distinct forms (6,7), one of which has recently been isolated(3) and synthesized(8). This compound, an amide of pantothenic acid and β-mercaptoethylamine, exists both in a reduced or thiol form (*pantetheine*) and as the corresponding disulfide (*pantethine*). Both forms show indistinguishable activity for *Lactobacillus helveticus* 80(8).

The ready interconversion of pantetheine and pantethine suggested the possible occurrence of mixed disulfides with LBF activity formed according to equation 1, below:

$$\text{PSSP} + \text{RSH} \rightleftarrows \text{PSH} + \text{RSSP} \quad (1)$$

where PSSP represents pantethine, RSH a mercaptan, PSH pantetheine, and RSSP a mixed disulfide. Examination of this possibility forms the subject matter of this article.

Experimental. The formation of mixed disulfides would be favored, if RSH in equation 1 were employed in excess, and the resulting mixture of PSH and excess RSH then reoxidized with iodine. Accordingly, synthetic pantethine (800 μg) was mixed in water with a 20-fold excess of the reducing compound, the solution diluted to 50 ml, and allowed to stand for 3-4 hours. The mixture was then either chromatographed directly (Na_2S or $Na_2S_2O_5$ as reducing agents) or oxidized with iodine to the disulfides(9), neutralized, and chromatographed. For the latter purpose, 0.005 ml (equivalent to about 5 LBF units) of each reaction mixture was spotted near one end of individual 1 x 30 cm strips of Whatman No. 1 filter paper. After drying, these were first equilibrated, then developed by the ascending technic, with water-saturated *n*-butanol. Active zones were located by the bioautograph procedure of Winsten and Eigen (10). *L. helveticus* 80 was used as the test organism with the pantethine-free medium previously described for this organism(5), but solidified with 1.5% of agar.

Mixed disulfides from pantethine. The results (Fig. 1) show that treatment of pantethine with any of the indicated reduced sulfur compounds always resulted in the formation of an active zone differing greatly in R_F value from that due to unchanged pantethine. Controls in which the thiol compounds were first oxidized to their disulfides, then added to pantethine, allowed to stand, and chromatographed, showed only one active zone corresponding to pantethine. Whereas pantethine itself is ninhydrin-negative, chromatograms of the reaction product of pantethine with β-mercaptoethylamine showed a ninhydrin-positive zone that corresponded exactly in outline and R_F value with the "extra" growth-promoting zone revealed by the bioautograph (Fig. 1G).† This behavior would be expected of a mixed disulfide containing a β-mercaptoethylamine residue as one component, since the latter fragment furnishes a ninhydrin-reactive amino group. Thus mixed disulfides are readily formed from pantethine in accordance with equation 1, and these mixed disulfides retain LBF activity. Since many thiols occur naturally (*e.g.* homocysteine, cysteine, cysteine peptides, proteins, glutathione, hydrogen sulfide, ergothioneine, etc.) in amounts far surpassing pantethine, similar mixed disulfides could readily be formed in natural materials in numbers limited only by the number of naturally occurring thiol compounds.

Discussion. In view of the fact that mixed disulfides are readily prepared and stable substances(11), it seems curious that the possible

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† Because of the comparative sensitivities of the growth and ninhydrin tests, larger amounts of the reaction product (up to 1000 units) must be used when the latter procedure is applied.

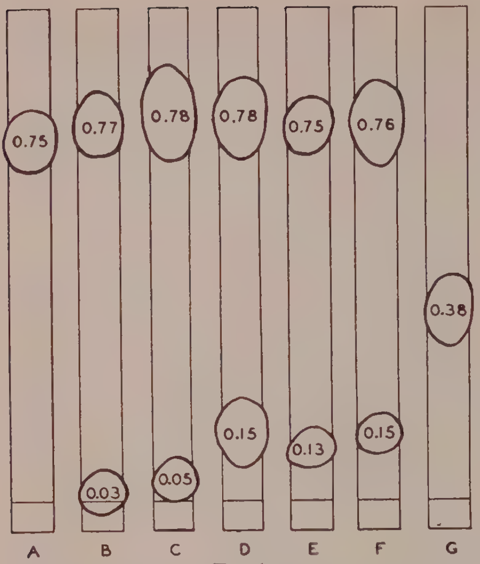

FIG. 1

Tracing of bioautographs showing the formation of mixed disulfides from pantethine and thiol compounds. R_F values of active zones are given within the zone area. A. pantethine, B. pantethine + reduced glutathione, C. pantethine + thiomalic acid, D. pantethine + cysteine, E. pantethine + sodium sulfide, F. pantethine + sodium metabisulfite, G. pantethine + β-mercaptoethylamine. See text for treatment.

natural occurrence of similar low molecular weight compounds has not been emphasized in the past. Although the number of such disulfides that might be formed from pantetheine and other naturally-occurring thiols more than suffice to explain the number of LBF-active zones so far detected in chromatograms of natural materials, it is quite possible that structures other than these may also contribute to the LBF activity of natural materials. From postulated structures for coenzyme A(12,13) and the relationship of the latter to LBF(14), it appears probable that structures intermediate in complexity between pantetheine and coenzyme A may exist, and contribute to the LBF activity of such digests. The occurrence of 3 zones with LBF activity in chromatograms from phosphatase-treated coenzyme A concentrates has been reported(7).

Repeated trials have shown that treatment of appropriate fractions of yeast extract with cysteine, followed by chromatography as described above, results in a pronounced shift in R_F values of certain of the LBF-active zones originally present. This preliminary evidence supports the idea that mixed disulfides contribute to the LBF activity of natural materials.

The possibility that coenzyme A may likewise occur naturally in several forms due to mixed disulfide formation should be considered.‡ The formation of such a disulfide with glutathione, for example, might possibly explain the high glutamic acid content of concentrates of a growth factor (PAC) for *Acetobacter suboxydans*(15), the growth-promoting action of which is duplicated by coenzyme A itself(16).

Summary. Treatment of pantethine with sodium sulfide, cysteine, glutathione, β-mercaptoethylamine, or several other thiols followed, in some cases, by oxidation with iodine resulted in formation of a series of new compounds with growth-promoting activity for *Lactobacillus helveticus* 80. These compounds appear to be mixed disulfides. The natural occurrence of similar mixed disulfides formed from pantetheine and other naturally occurring thiols may explain in part the presence of several chromatographically distinct forms of LBF in natural materials.

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‡ Lipmann and coworkers (private communication) have shown independently that mixed disulfides of coenzyme A with other thiols may exist.

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Effect of Cortisone and ACTH During Sensitization on Eosinophils and Anaphylaxis in Guinea Pigs. (18704)

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Leger, Leith and Rose(1) have shown that the administration of ACTH to sensitized guinea pigs 6 to 8 hours prior to the giving of a challenging injection of the antigen does not alter the anaphylactic reaction. This observation has been confirmed by Friedlaender and Friedlaender(2) and by Dworetzky, Higgins and one of us (Code)(3). It has also been demonstrated that treatment with cortisone just prior to the administration of the antigen likewise has no observable effect upon the ensuing reaction(3,4). While these investigations clearly showed that cortisone and ACTH are without visible effect upon the anaphylactic reaction in fully sensitized guinea pigs, the possibility remained that ACTH and cortisone might have some influence upon the development of the hypersensitive state if they were administered with the sensitizing injections. This possibility was further heightened by the observations of Germuth and Ottinger(5), that the administration of cortisone and ACTH during the administration of sensitizing injections to rabbits inhibits the development of the Arthus phenomenon and the appearance of free antibodies in the blood.

Methods. Two series of guinea pigs were studied. In both series there were 3 groups of animals, each composed of an equal number of males and nonpregnant females. The initial body weights of all the animals ranged from 350 to 490 g and there was no significant difference between the initial mean body weight of the animals in the different groups. All the animals were sensitized with diluted fresh egg white. This was prepared by mixing one part of the whites of fresh eggs with 4 parts saline solution and discarding any insoluble material. The schedule of injections was identical in both series. Commencing on the third or the fourth day after the administration of cortisone or ACTH, each guinea pig was given 4 successive daily subcutaneous injections of 0.5 ml. The sensitivity of the animals to the antigen was tested three to seven weeks later by the intravenous administration of 0.2 ml or less of the diluted egg white.

In Series 1 the 3 groups were composed of 12 guinea pigs each. The animals in the first group served as controls and were not given injections of either hormone. Those in the second group were given 25 mg of cortisone in a single injection, and then 4 days later 5 mg and thereafter 5 mg every other day for 27 days. The sensitivity of 4 of the pigs to egg white was then tested. Four of the remaining 8 animals continued to receive cortisone, 4 were untreated and at the end of 14 more days the sensitivity of these pigs was tested. The animals in the third group received ACTH.* Initially, each was given an injection of 6.25 mg repeated 12 hours later and thereafter 1.25 mg every 12 hours for 27 days, at the end of which the sensitivity to egg white of 4 of the group was tested. The remaining 8 received no further treatment and their sensitivity to egg white was tested 14 days later. The sensitivity of 4 of the control pigs was also tested at 27 days and the remainder 14 days later. Series 2

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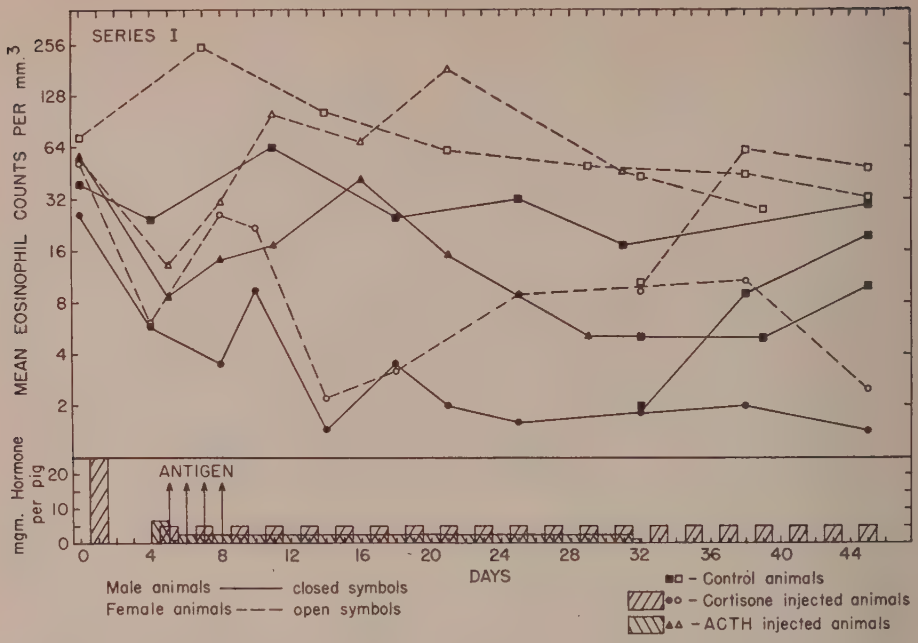

FIG. 1

Effect of the prolonged administration of ACTH and cortisone on eosinophils in guinea pigs in Series 1 of this study.

likewise consisted of 3 groups, each composed of 8 guinea pigs. Half of the control group received 0.2 ml of saline solution twice a day during the injection period. The remainder of the controls were untreated. The cortisone-treated group received 10 mg daily for 14 days. Those receiving ACTH were given 2.5 mg every 6 hours for 6 days; then 5 mg every 6 hours for 6 days. The sensitivity of all of the guinea pigs in series 2 was tested by an intravenous administration of the antigen 53 days after the first sensitizing injection.

As measures of the action of ACTH and cortisone, the number of eosinophils in the blood and the body weights of the animals were determined periodically. The eosinophils were counted by the modification of Dunger's (6) method which was developed by Thorn, Forsham, Prunty and Hills(7).

Results. Body weights. While the animals were receiving injections, all but 2 gained weight. In series 1 the control animals gained, on the average, 143 g; and those receiving cortisone or ACTH gained 92 g. During the shorter injection period in series 2, the uninjected control animals gained, on the average, 75 g, the injected controls 40 g, those receiving cortisone 20 g and those given ACTH 15 g.

Eosinophil counts. A single control eosinophil count was made in the majority of animals prior to the injection of saline solution or hormone. The mean number of eosinophils per cubic millimeter of blood in the males was 32 ± 4.9 and 56 ± 7.3 in the females. The probability of a difference as great as that between these means occurring by chance is less than 1 in 100. When 1.25 mg of ACTH was given every 12 hours, as in the third group of series 1, no decisive change was observed in the number of eosinophils in the peripheral blood. When, however, larger doses (2.5 g) were given every 6 hours, as in series 2, a decisive and sustained reduction

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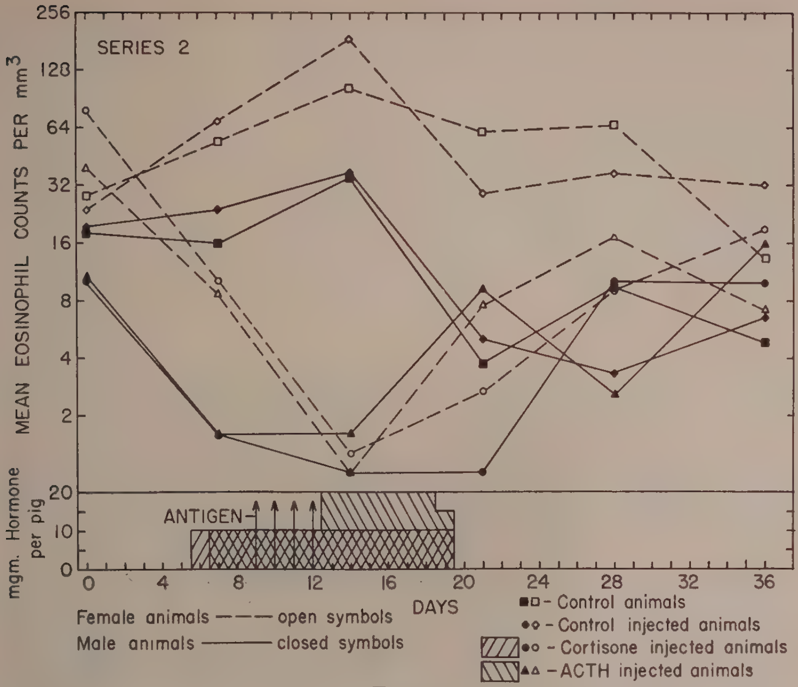

FIG. 2
Effect of repeated large doses of ACTH and cortisone on eosinophils in guinea pigs in Series 2 of this study.

in the number of circulating eosinophils occurred in both males and females (Fig. 1 and 2). Cortisone produced a pronounced eosinopenia in both series of animals (Fig. 1 and 2).

Anaphylactic shock. All the animals in both series exhibited severe reactions immediately after the antigen was given intravenously. The symptoms in every instance were characteristic of anaphylactic shock. Not more than 1 animal in any group survived the immediate effects of the reaction. All those which died promptly, exhibited the voluminous emphysematous lungs so characteristic of anaphylaxis in guinea pigs.

Comment. The observation of Dworetzky, Higgins and one of us (Code)(3) that the number of eosinophils in the blood of female guinea pigs is higher than in males has been confirmed. Likewise, their finding that both cortisone and ACTH produce eosinopenia in male and nonpregnant female guinea pigs has been verified.

Neither cortisone nor ACTH had any observable effect upon the anaphylactic reaction in guinea pigs when these hormones were given during the period of sensitization. The conclusion may be drawn that guinea pigs sensitized by subcutaneous injections show a different response to injections of ACTH and cortisone than rabbits sensitized by intracutaneous injections, as in the experiments of Germuth and Ottinger(5). Likewise, there is apparently a species difference between the guinea pig and the mouse, since Nelson, Fox and Freeman(8) have shown that the administration of cortisone to sensitized mice eighteen hours before the intravenous injection of the challenging dose of antigen prevents or diminishes the severity of the reactions. No satisfactory reason for these

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species differences can be offered on the basis of the results of these experiments.

Summary. Two series of 36 and 24 guinea pigs were studied. Cortisone was given to a third of the animals, ACTH to a third and the remaining third served as controls. The administration of the hormones, in moderate or large doses, was started before giving the sensitizing injections of egg white, and was continued while they were being given and for varying periods after their completion. No treatment had any effect on the severity of anaphylactic reactions induced by the subsequent intravenous injection of the antigen. Used in adequate amounts, both hormones produced pronounced and sustained reductions in the number of circulating eosinophils in the blood of male and nonpregnant female guinea pigs.

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Negative Effects of Vitamin B_{12} on Blood of Healthy Individuals, with Special Reference to Eosinophils.* (18705)

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While examining blood smears from patients ill with pernicious anemia, who responded to therapy with vit. B_{12}, we were surprised to find that there had been no specific increase in eosinophilic polymorphonuclear leucocytes. We had expected to find a rise in eosinophilic cells since it is well known that oral or intramuscular therapy with whole liver or with liver extracts commonly produces an eosinophilia in patients ill with pernicious anemia or in healthy individuals(1-9), which may reach as high as 75% of the total white blood cell count. Because of this, observations were made on the effects of therapy with relatively large amounts of vit. B_{12} given, either by mouth or by injection, to 3 healthy young adults and to 5 healthy children.

Methods. Three adult males and 5 school children served as subjects. They were healthy and none of them gave a history of previous allergic disease, anemia, or blood dyscrasia. In the adults, studies of their blood were made 3 times a week; during a control period of 2 weeks, during a 28 day vit. B_{12} therapy period, and during the 2 weeks of the post-therapy period. Blood from the children was observed twice during the pre-therapy period and at weekly intervals during the therapy and post-therapy periods. Fasting blood was collected in heparinized tubes and from these determinations of hematocrit, hemoglobin, and red blood cell counts were made by standard technics. Absolute eosinophil counts and white blood cell counts were made by a modification of Randolf's technic using 3 parts of phloxine to one part of methylene blue(11). Differential counts of stained blood were made by standard technics using fresh blood cover-glass smears and counting 200 white blood cells.

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TABLE I. Absolute and Differential Counts of Eosinophiles in 8 Healthy Persons before and after Treatment with vit. B_{12}.

Subject	Sex	Age	B_{12} µg per day	Absolute Eosinophile Counts per mm³				Differential Count of Eosinophiles: % Total White Blood Cells			
				Pre Vit. B_{12}		Post Vit. B_{12}		Pre Vit. B_{12}		Post Vit. B_{12}	
P.	M	11	10*	136	176	239	220	2	2	3	2
W.	M	7	10†	116	132	117	83	2	1	1	1
H.	M	10	10†	156	242	261	225	4	6	4	3
D.	M	8	10†	117	99	75	77	1	1	1	2
H.W.	F	12	10†	50	77	89	84	2	1	2	0
C.	M	20	300†	176	149	193	187	1	2	2	4
L.	M	27	300†	321	308	385	264	1	3	3	2
M.	M	28	75*	303	396	297	401	1	3	1	2

* Intramuscular therapy.
† By mouth.

The dosage of vit. B_{12} was regulated to provide approximately similar amounts of crystalline B_{12} to that present naturally in 1½ lb of raw calves liver(12). Accordingly, each of 2 adults received 300 µg vit. B_{12} a day by mouth for 28 days, and the third subject was given 75 µg of vit. B_{12} a day by intramuscular injection for the same period of time. The children received 10 µg of the vitamin each day for 28 days; 4 took this by mouth and the fifth received it by intramuscular injection.

Results and discussion. It is apparent that at least two biochemical mechanisms associated with liver therapy stimulate an increase in circulating eosinophils. The first type of liver eosinophilia is the result of sensitization to liver protein or to the protein of the animal from which the liver or liver extract was obtained. This commonly produces clinical manifestations of sensitization in the patient receiving the liver(6). The second type of liver eosinophilia is a specific effect of liver therapy and occurs uniformly, even in healthy individuals, following the ingestion of 1½ lb of raw fresh calves liver for 28 days(9). This latter type of eosinophilia was commonly seen in patients ill with pernicious anemia, who lived on raw liver, in the years immediately following Minot and Murphy's(1), discovery of the value of a diet high in liver for treatment of the disease. This eosinophilia is symptom free and is not associated with clinical manifestations of allergy.

Vit. B_{12} did not produce an eosinophilia in our subjects (Table I). Clinically there was no evidence of development of toxic or allergic disturbances in either adults or children, who remained healthy throughout the period of study. Hematologic data obtained in the control period were not altered significantly in any subject following therapy. The data, as far as the children are concerned, are somewhat at variance with those obtained by Wetzel *et al.*, who found an 8% eosinophilia in children after treatment with vit. B_{12}(10). The small rise in the eosinophils reported by Wetzel may be a normal variation when making differential counts of cells from slides(13), which is not as statistically valid as counts made by direct counting technics(14).

Summary. 1. Oral or intramuscular administration of large amounts of vit. B_{12} was without effect on the blood of healthy adults and children. In particular, an eosinophilia was not observed. 2. Neither allergic nor toxic symptoms were noted during or after therapy. 3. It is concluded that vit. B_{12} is not the eosinophilic factor present in raw calves liver.

We wish to thank Dr. Augustus Gibson, Jr. of Merck and Co., Rahway, N. J. for supplies of vit. B_{12}.

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Failure of Vitamin B_{12} to Modify Goitrogenic Action of Thiouracil. (18706)

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Meites(1) has recently made the interesting observation that vit. B_{12} administered in doses of 40-80 μg per kilo of food markedly inhibited the development of goiter in thiouracil-fed rats. In view of the importance of this finding similar experiments were undertaken in this laboratory to confirm these results.

Experimental. In the first experiment, twenty-four 50 g male albino rats were divided into 4 equal groups. The animals were fed a basal diet of Purina mink chow to which 0.03% propylthiouracil and an aqueous solution of vit. B_{12} were variously added as indicated in Table I. The experiment was continued for 24 days when the animals were killed with chloroform and the thyroids dissected and weighed. The results are summarized in Table I. It can be seen that no appreciable inhibition of goitrogenesis was produced by the administration of vit. B_{12}.

In view of these results, a second experiment was carried out, duplicating Meites' conditions as exactly as possible. Forty 50-60 g male albino rats were divided into 4 equal groups and fed a basal diet consisting of: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; whole milk powder, 20%; alfalfa leaf meal, 6%; brewers yeast, 3%; and table salt, 1%. Crystalline vit. B_{12} as a triturate of sodium chloride[†] and

TABLE I.

Group	No. of animals	Diet	Avg Body Wt		Avg Thyroid Wt	
			Orig., g	Final, g	Actual, mg	mg/100 g Body Wt
1	6	Basal	52.7	120.3	10.1	8.7 ± 0.58[*]
2	6	Basal + 80 γ B_{12}/kg food	52.0	128.4	9.8	7.6 ± 0.67
3	6	Basal + .03% propylthiouracil	49.8	79.6	34.3	43.1 ± 4.65
4	6	Basal + .03% propylthiouracil + 80 γ B_{12}/kg food	55.0	87.5	37.6	41.8 ± 3.28

[*] Stand. error of mean = $\sqrt{\dfrac{\Sigma d^2}{n\,(n-1)}}$.

TABLE II.

Group	No. of animals	Diet	Avg Body Wt		Avg Thyroid Wt	
			Orig., g	Final, g	Actual, mg	mg/100 g Body Wt
1	10	Basal	53.6	134.3	11.3	7.9 ± 0.35
2	10	Basal + .03% propylthiouracil	59.5	96.0	38.9	40.5 ± 2.75
3	10	Basal + .03% propylthiouracil + 40 γ B_{12}/kg food	56.3	90.6	44.1	49.4 ± 3.61
4	10	Basal + .03% propylthiouracil + 80 γ B_{12}/kg food	57.4	84.3	38.9	47.3 ± 3.93

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[*] U. S. Public Health Service Postdoctoral Research Fellow, National Cancer Institute.

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[†] Generously supplied through the courtesy of Dr. Leo V. Curtin of Merck and Co.

0.03% propylthiouracil were variously added as indicated in Table II. At the end of 30 days the animals were killed with chloroform and the thyroids weighed. In this experiment there was again no inhibition of the size of the goiters produced by thiouracil in the B_{12}-fed animals. In both experiments, the thyroids of the B_{12}-fed animals were just as large, vascular, and friable as those of the controls. No satisfactory explanation is in evidence to account for the discrepancy in findings between the two laboratories.

Summary. It was not possible to confirm the antigoitrogenic effect of vit. B_{12} in thiouracil-fed rats.

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Urinary Excretion of Neutral 17-Ketosteroids by Normal Dogs. (18707)

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The Zimmermann reaction with *m*-dinitrobenzene is used with considerable success for the assay of neutral 17-ketosteroids in human urine. Engstrom and Mason(1) and Talbot and coworkers(2) have shown that errors in the colorimetric determination caused by the presence in urine of colored substances and chromogenic material other than 17-ketosteroids may be eliminated either by a preliminary Girard separation or, if the reaction is carried out according to the technic of Callow *et al.* (3), by application of a color correction equation. Both methods give essentially the same results. There have been few reports in which this technic has been applied to the urine of experimental animals. Davis *et al.* (4) and Kimeldorf(5) used it for rabbit urine, Henriques *et al.*(6) for Cebus monkey and Dorfman *et al.*(7) for chimpanzee urine. Dog urine was analyzed for 17-ketosteroids by Paschkis and coworkers(8) and by Lawrence (9). In both cases crude urine extracts were used and no correction for overestimation due to non-ketosteroid chromogens was given.

We have analyzed the urine of normal dogs for neutral 17-ketosteroids, using both the isolation technic with Girard's reagent(10) and the color correction equation(11) and obtained values consistently lower than Paschkis(8) and Lawrence(9). Moreover, spectrochemical evidence indicates that even these values may be too high.

Method. Normal mongrel dogs were kept in metabolism cages and their urine was collected in 3 ml concentrated HCl as a preservative. The urine was stored in the refrigerator and analyzed within 48 hrs. after collection. Each urine sample was made up to 1 liter, acidified to 15 vol. % with concentrated HCl and hydrolyzed by boiling for 10 min. Ten g NaCl was added while hot, the urine was immediately cooled to 60°C and transferred to a modified Hershberg-Wolfe extractor(12). One 1 CCl_4 was used, 500 ml in the extractor and 500 ml in the boiling flask. The urine was extracted for exactly 1.5 hours and the extract purified and analyzed according to Talbot's method

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TABLE I.
Urinary Excretion of Neutral 17-Ketosteroids by Normal Dogs, mg/24 hr.

Dog. No. & Sex	No. of Specimens	Crude Extract	Corrected Value	Ketonic Fraction	K_S
13 ♀	4	1.2	.4	.3	1.1
19 ♀	2	1.1	.6	.6	1.6
29 ♀	2	0.8	.5	.4	1.5
30 ♀	2	0.6	.6	.6	1.2
20 ♂	2	1.5	.5	.4	1.1
58 ♂	2	1.5	.6	.7	1.7
64 ♂	2	1.5	.6	.5	1.4

(10). After taking aliquots for the analysis of the crude urine extract, the remainder was evaporated to dryness and the residue fractionated with Girard's reagent-T(10). Following reaction with *m*-dinitrobenzene, the crude extract, ketonic and non-ketonic fractions were read on a Coleman Universal Spectrophotometer, Model 14, at 520 mμ and then at 420 mμ. All analyses were carried out in duplicate, 2 samples of standard solution of dehydroisoandrosterone* and 3 blanks being included in every series of analyses.

Results. The spontaneous 24-hr. urinary excretion of neutral 17-ketosteroids by normal dogs is given in Table I. All values are the mean of at least two 24-hr. specimens. The values from crude urine extracts were corrected by use of the Gibson-Evans equation (11). Corrected $E_G = \frac{K_I E_G - E_V}{K_I - K_S}$, where E_G and E_V are the extinctions at 520 mμ and 420 mμ, respectively, $K_I = 1.7$ from the mean ratio of the extinction at 420 mμ to the extinction at 520 mμ for the non-ketonic fraction in 47 measurements on 10 normal dogs, and $K_S = 0.4$ from the same ratio of extinctions for crystalline dehydroisoandrosterone. The K_S values in the table are extinction ratios for the ketonic fractions and show a large deviation from the K_S value of pure 17-ketosteroid. Nevertheless, there is good agreement between the 17-ketosteroid concentration determined in the ketonic fraction and the value calculated by applying the correction equation to the crude urine extract.

The high K_S values for the ketonic fractions of dog urine led to an investigation of the absorption spectra of the colored products of the Zimmermann reaction. Fig. 1 shows a comparison of the spectra given by the products of the *m*-dinitrobenzene reaction of different dog urine extracts with that of dehydroisoandrosterone and human urine extracts. Only the crude extract from dog urine to which 5 mg dehydroisoandrosterone per 1 had been added shows the absorption maximum at 520 mμ, characteristic for 17-ketosteroids. In contrast to human urine extracts, which exhibit an absorption peak at 520 mμ, neither the crude extract nor the ketonic fraction of dog urine give the characteristic color reaction.

The analytical method was tested by parallel determinations with the addition of pure ketosteroid to normal urine. Table II gives

TABLE II.
Recovery of 5 mg Dehydroisoandrosterone Added to Human and Dog Urine, in mg.

Urine	DHA Added	Crude Extr.	Corrected Value	% Recovery
Pooled human ♂	—	16.0	11.8	
,, ,,	+	21.0	16.8	100
24-hr dog ♀	—	1.3	0.5	
,, ,,	+	6.5	5.8	106
,, ,,	—	1.8	1.0	
,, ,,	+	7.8	6.4	108

TABLE III.
Urinary Excretion of Neutral 17-Ketosteroids by Normal Dogs after ACTH, in mg/24 hr.

Dog. No. & Sex	Treatment	Crude Extract	Corrected Value	Ketonic Fraction	K_S
19 ♀	Pretreatment	1.4	.6	.7	0.9
,,	25 mg ACTH	0.8	.3	.4	0.8
,,	Posttreatment	1.2	.3	.5	0.9
30 ♀	Pretreatment	1.1	.5	.6	1.3
,,	25 mg ACTH	2.3	.6	.7	1.6
,,	,, ,,	1.0	.5	1.0	1.5
,,	,, ,,	1.0	.5	.6	1.2
,,	Posttreatment	0.8	.3	.7	1.2

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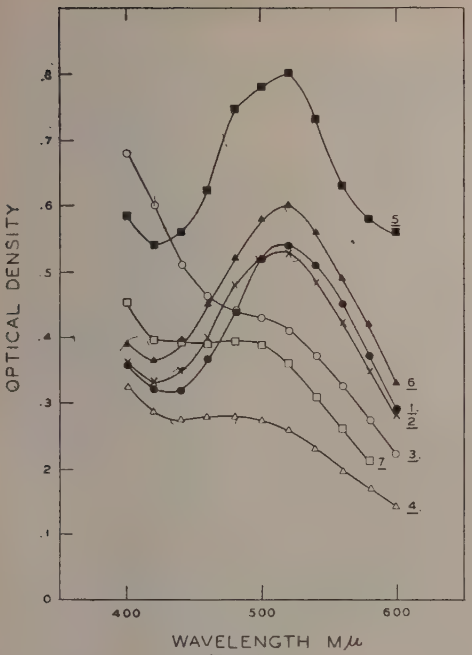

FIG. 1

Absorption spectra of urine extracts after Zimmermann reaction.

1. Dehydroisoandrosterone (DHA); 2. dog urine + DHA, crude extract; 3. crude extract of dog urine; 4. ketonic fraction of dog urine; 5. crude extract of human urine; 6. ketonic fraction of human urine; 7. crude extract of urine from ACTH-treated dog.

the results of recovery experiments on human and dog urines. To a 1 liter aliquot of pooled normal human male urine and to aliquots of two 24-hr. urine specimens from normal female dogs 5 mg dehydroisoandrosterone were added. The values from the crude urine extracts were corrected by use of the Gibson-Evans equation, uring $K_I = 1.0$ for the human urine. This value was obtained as the mean of 20 analyses of urine samples from 10 different human subjects. Recoveries of added ketosteroid were uniformly good.

The ketosteroid excretion of the normal dog after adrenal stimulation with adrenocorticotrophic hormone is recorded in Table III. Two female dogs were given 25 mg ACTH per day intramuscularly in 3 equal doses at 3-hr. intervals. One dog was treated for one day, the other for 3 days. The urine was collected on the day prior to, during, and following injection and analyzed as before. No significant change in the urinary excretion of ketosteroids occurred, nor was there any change in the absorption spectrum of the urine extract of a treated dog after reaction with *m*-dinitrobenzene (Fig. 1).

Discussion. The evidence presented indicates that the normal dog excretes only negligible quantities of neutral 17-ketosteroids. The higher values reported by Paschkis and coworkers (0.5-1.5 mg/day) and by Lawrence (0.2-5.4 mg/day) are undoubtedly due to interference of urinary pigments and chromogens other than 17-ketosteroids with the colorimetric assay. Urine extracts purified with Girard's reagent do not give the characteristic color reaction and even the low values given in the present paper are probably too high. In confirmation of Paschkis *et al.*(8) no sex difference was found in the ketosteroid excretion of dogs. Also in contrast to humans, dogs did not respond to adrenal stimulation by ACTH with an increase in urinary ketosteroids. This was also observed by Lawrence (9), but no data were given. Furthermore, dogs do not respond to the administration of testosterone by increased urinary excretion of androgens, as shown by Kochakian(13), by Dingemanse and Tyslowitz(14) and by Paschkis *et al.*(8). The explanation for the negligible excretion of ketosteroids may be that dogs efficiently convert androgens to estrogens(8).

Summary. 1. Normal dogs excrete less than 1 mg neutral 17-ketosteroids per day. 2. No sex difference exists in the urinary ketosteroid excretion of dogs. 3. Administration of ACTH to dogs does not result in increased excretion of ketosteroids.

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Histoplasma capsulatum in Tissue Culture.* (18708)

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In this laboratory certain tissues of the horse have been used in tissue culture to demonstrate infection with the virus of equine abortion(1). During the course of these studies, intracellular organisms identical in appearance with *Histoplasma capsulatum* were observed in certain horse tissues grown on film-coated cover-glass cultures. Details of the morphological study of this naturally infected horse tissue will be presented elsewhere(2).

Since no studies on growth of *H. capsulatum* (or other pathogenic fungi) in *in vitro* cultures of animal tissues have been reported, it seemed of interest to attempt the experimental cultivation of this fungus in tissue cultures of horse and chick embryo tissue. The results of this study are presented here.

Experimental Procedures. Horse tissues were aseptically obtained from a mare at 11 months gestation. Amnio-allantoic membrane and placenta were cultivated in flasks(3) and on cover-glasses placed in test tubes. Chick embryo tissue was cultivated by the latter method only. (The choice of the particular horse tissue used was determined by the findings in the naturally infected animal). Chick embryo extract (E.E.) and chicken plasma were prepared by conventional methods. The supernatant or liquid phase consisted of 50% human ascitic (cancer) fluid, 47% Hanks' balanced salt solution (B.S.S.), and 3% E.E., with the pH of the mixture adjusted to 7.6. Tissues from flask cultures were fixed in Zenker-acetic acid, and H and E stained paraffine sections prepared from them. Cover-glass cultures were fixed in Zenker-acetic acid, and stained by the hematoxylin-eosin-azure method.

The following methods were used in preparing tissue cultures of horse amnio-allantoic membrane and placenta, and 12-day chick embryo muscle. The tissues were cut into 2 mm squares and immediately rinsed thoroughly with B.S.S. The rinsed fragments were then placed in 0.5 ml of B.S.S., to which was added 0.5 ml of a yeast cell suspension of *H. capsulatum* (Vanderbilt University strain No. 603) containing 30 million yeast cells per ml. The mixture of tissue fragments and organisms was then allowed to stand for two hours at 5°C. *Flask method.* Only horse tissues were used in this method. Two parallel sets of flasks were prepared, one series with amnio-allantoic membrane, and one with placental tissue. Three to four pieces of tissue, with a minimal amount of fluid, were transferred with a 5 ml pipette to a 50 ml Erlenmeyer flask containing 3 ml of supernatant, incubated at 37°C, and observed daily. Tissues were fixed for sectioning after 5 and 8 days of incubation. *Cover-glass method.* Three or 4 pieces of tissue were embedded in a plasma clot (equal parts of chicken plasma and E.E.) on a No. 1 12 x 50 mm cover-glass. This preparation was then introduced into a 16 x 150 mm pyrex test tube, and after standing for one hour, 2 ml of supernatant was added to each tube, and the cotton plug replaced with a rubber stopper. The cultures were slanted in a test tube basket at an angle which allowed the supernatant barely to cover the surface of the cover-glass, and incubated at 37°C. The following controls were prepared in the same manner: (1) tissue fragments without *H. capsulatum,* and (2) *H. capsulatum* without tissue fragments. All cultures were examined daily. The experiments dealing with placenta and amnio-allantoic membrane were terminated after five days of incubation; the chick embryo tissue experiments were terminated after 3, 4, 5 and 6 days of incubation.

Results. Flask method. Examination of H and E stained preparations of placenta and

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* This investigation was supported in part by a grant from the Grayson Foundation.

1. To be published.

2. To be published.

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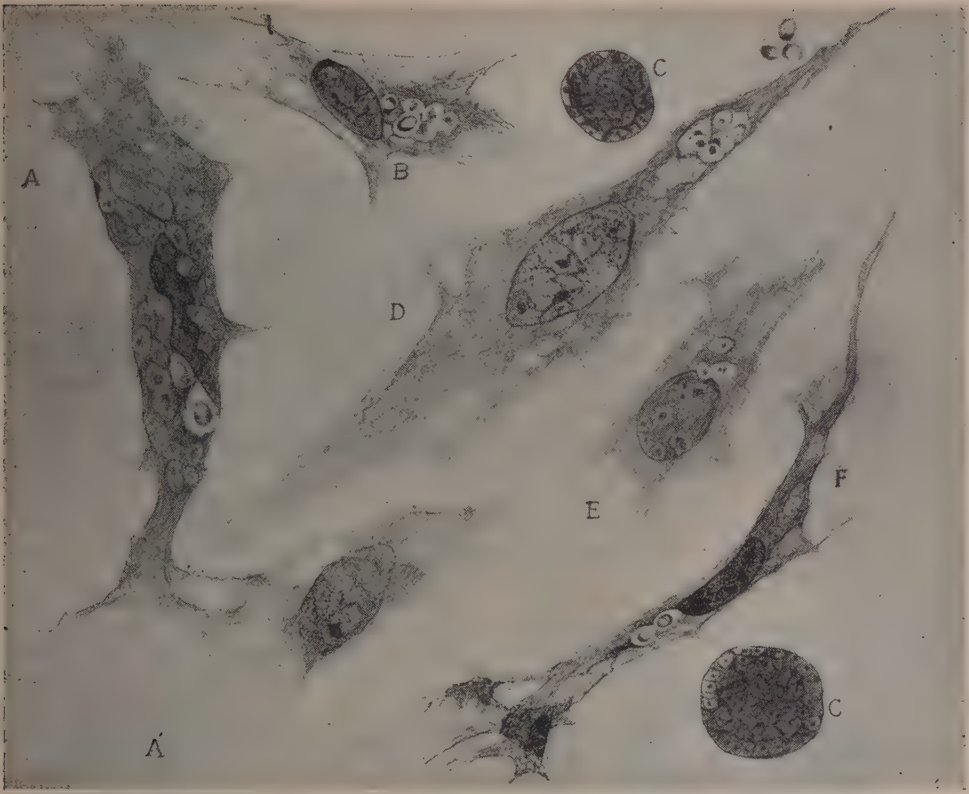

FIG. 1

Five-day-old cultures of 11-month-old horse placenta. A, B, E and F are fibroblast-like cells of the outgrowth containing yeast cells of *Histoplasma capsulatum*. A′ is a normal cell included for comparison. C represents a highly diagrammatic sketch of the sac type of colony of *H. capsulatum*. (Photograph of camera lucida drawing; x1500.)

amnio-allantoic membrane revealed innumerable yeast cells of *H. capsulatum* multiplying within the interstices of the connective tissue. No intracellular yeast cells could be demonstrated unequivocally. The supernatant contained a heavy growth of yeast cells of the fungus; no mycelial development was noted.

Cover-glass method. In previous tissue culture experiments by the cover-glass method, with horse placenta and amnio-allantoic membrane of 6, 9 and 10 months gestation, it was found that the placental tissue produced only a scanty outgrowth of cells, whereas the amnio-allantois gave striking growth. In the present experiment with 11-month-old horse placenta, both in the presence and in the absence of *H. capsulatum*, the amount of outgrowth was commensurate with that obtained with younger placentas, and in this instance resembled fibroblasts. With 11-month-old amnio-allantoic membrane, however, no cells grew or migrated from any of the tissue fragments. Stained preparations of both chick embryo tissue and horse placenta, regardless of the age of the culture, revealed scattered fibroblasts or fibroblast-like cells with few to many intracellular yeasts (Fig. 1 and 2). The infected cells appeared without stigmata of degeneration.

Of particular interest in these cover-glass preparations was the striking form assumed by many of the colonies of *H. capsulatum* growing in the plasma clot. Scattered throughout the clot were numerous globose sacs con-

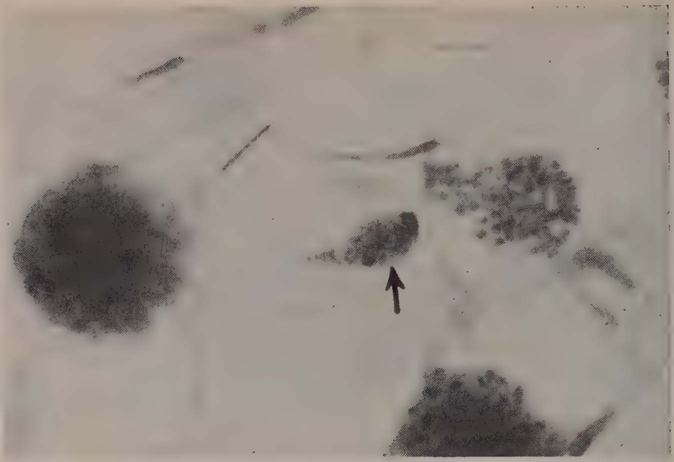

FIG. 2.

Five-day-old culture of 12-day-old chick embryo leg muscle. Intracellular yeast cell forms are indicated by an arrow. Note the compression of the cell nucleus. The large, dark-staining masses are the sac type of colony of *H. capsulatum* (see text and Fig. 3). Many cells of the explant are out of focus due to the thickness of the preparation. x800.

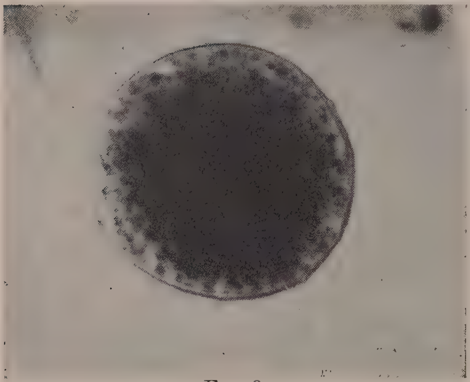

FIG. 3.

Sac type of colony of *H. capsulatum*, with yeast cells enclosed within a well-marked membrane. From a 5-day-old culture of 11-month-old horse placenta. x 400.

taining myriads of encapsulated yeast cells surrounded by a well-defined, sharply-delimiting membrane (Fig. 3). These sacs, which bore a striking resemblance to the *in vivo* form of *Coccidioides immitis*, varied in size from 20 to 300 μ in diameter. Further simulation of the sporangia of *C. immitis* was seen in sacs which had burst, allowing their contents to scatter into the surrounding plasma (Fig. 2). Under these circumstances, the individual, characteristic, budding yeasts of *H. capsulatum* could be readily observed. Not all of the colonies of the fungus assumed this bizarre form; many were the usual flatly-convex colony, with no delimiting membrane. The sac type of colony was far more abundant in the cover-glass cultures containing tissue fragments than in the cultures lacking tissues. Growth of *H. capsulatum* in the mycelial form was negligible in the former type of preparation, marked in the latter. In neither type of preparation, however, was there any growth of organisms in the supernatant.

Discussion and conclusions. A study of chick embryo and horse placental tissue, when cultivated in tissue culture with *H. capsulatum* by the cover-glass method, has revealed that fibroblasts or fibroblast-like cells of the outgrowth contain yeast cells of this organism in the cytoplasm. It is of interest to note that sections of infected tissues from systemic histoplasmosis rarely, if ever, show yeast cells in fibroblasts. Whether or not these intracellular yeasts can continue to multiply within the fibroblast, or are ultimately destroyed, remains to be determined. Similarly, whether or not the fibroblast which contains yeast cells can divide further, or undergoes degeneration, is not yet known. An explanation for the unique type of colonial morphology of

H. capsulatum seen in the cover-glass cultures, cannot be given at this time. This phenomenon is not dependent upon the presence of living tissues, since the sac-like type of colonies were observed in the controls which lacked tissue fragments. Of possible significance is the fact that this type of colony occurred only when the inoculated yeast cells were fixed *in situ* and surrounded by clotted plasma; in the supernatant of the flask cultures, only free, individual yeast cells were seen. Further work is in progress in an attempt to clarify these problems.

ADDENDUM. Since submitting this paper for publication an article by O. H. Duque has come to our attention. Writing in *Bol. Clin., Univ. Antioquis,* 1937, v9, 312, he reports the results of the cultivation of several pathogenic fungi with fibroblasts grown *in vitro*. The only positive findings concerned a culture inoculated with the mycelial phase of *H. capsulatum* which developed intracellular bodies interpreted as spores.

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Effects of Cortisone and Adrenocorticotrophic Hormone on Wound Healing in Normal and Scorbutic Guinea Pigs. (18709)

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Evidence suggests that ascorbic acid may influence the secretory activity of the adrenal cortex(1,2). However, the rôle of this vitamin in adrenal metabolism is still obscure. A possible mode of action was indicated by Lowenstein and Zwemer(3), who isolated an ascorbic acid-adrenal corticosteroid conjugate. Their work is yet to be confirmed. Symptoms of adrenocortical insufficiency resemble those of scurvy(4). Both conditions are characterized by hypersensitivity to stress(5) and may be favorably modified by adrenocortical hormone or by ACTH(4,6-9). Furthermore, increased utilization or destruction of ascorbic acid has been observed in stress(10). Recent investigations have demonstrated that adrenal cortical hormone may inhibit the growth of connective tissue, thus simulating, in some respects, the pathologic changes seen in scurvy(11-13). Moreover, like ACTH and Cortisone, vit. C in massive doses(14) or in conjunction with desoxycorticosterone(15,16)

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TABLE I. Comparison of Findings in Hormone-Treated and Untreated, Normal and Scorbutic Guinea Pigs.

Diet	No. of animals	Group	Wounds			Avg adrenal/body wt ratio $\times 10^{-5}$	Avg No. eosinophils per mm^3 of blood	Hyperplasia (+) or atrophy (—) of the zona fasciculata	Avg conc. of adrenal ascorbic acid in γ/100 mg
			Hemorrhage	New formation of collagen	Fibroblastic proliferation				
Scorbutigenic + ascorbic acid	10	Control	+	+	++	20	55	±	126
	10	ACTH	+	+	++	33	0	3+	114
	8	ACTH vehicle	+	+	++	19	60	±	117
	16	Cortisone	+	+	++	16	0	—	86
	8	” vehicle	+	+	++	20	70	±	115
Scorbutigenic	10	Control	3+	—	+	31	0	3+	2±
	10	ACTH	3+	—	+	40	0	4+	2±
	10	ACTH vehicle	3+	—	+	28	0	3+	2±
	10	Cortisone	3+	—	+	19	0	±	6±
	8	” vehicle	3+	—	+	30	0	3+	5±

has been reported to benefit patients with rheumatoid arthritis.

This study was undertaken in an effort to elucidate the rôle of ascorbic acid in wound repair and in adrenal metabolism. The effects of scurvy upon wound healing in guinea pigs have been compared with those of ACTH and Cortisone, and the influence of these hormones upon the scorbutic process has been evaluated.

Experimental. One hundred guinea pigs, weighing 300 to 400 g, were divided into 10 groups, as outlined in Table I. Males and females were caged separately. All were fed a standard scorbutigenic ration(17), to which were added supplements of ascorbic acid, 20 mg daily *per os*, for those not intended to develop scurvy. Utilizing Wolbach's technic (18), each animal was surgically wounded, in order to secure an area in which formation of connective tissue might be studied. This procedure was carried out on the twelfth day of the scorbutigenic regimen. Beginning at the time of operation, hormones were administered as indicated in the table, ACTH[*] being injected subcutaneously, 1.0 mg per 100 g body weight daily, in divided doses at intervals of approximately 6 hours. This amount was doubled at the end of the first 5 days. Cortisone[†] was given daily in single subcutaneous injections, 4.0 mg per 100 g body weight. Five days after operation one-half of the animals in each group were sacrificed; the remaining half were killed on the tenth postoperative day. Each guinea pig was killed by a blow on the head and subsequent exsanguination from the carotid artery. One adrenal gland from each animal was assayed for total ascorbic acid by a modification of the method of Roe and Kuether(19). Total eosinophil counts by the method of Thorn (20), as well as differential blood counts, were performed at the time of death. Sections of the wounds were stained by hemalum and eosin, toluidin blue, the periodic acid-leukofuchsin reaction, the Mallory-Heidenhain azan procedure, the Wilder silver stain for reticulum, and by Gomori's method for alkaline phosphatase. Sections of adrenal glands were stained by hemalum and eosin, and, in addition, frozen sections of adrenal gland were

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[*] The ACTH used in these studies was provided by Armour and Co. Dosages are equivalent to Armour Standard LA-1-A.

[†] The Cortisone employed in this experiment was donated by Merck and Co.

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stained for neutral fat with oil-red-O.

Results. Our observations are outlined in Table I. (Histologic details are to be described in a separate report.) The wounds of the scorbutic animals showed in each instance a failure to heal and were characterized by a relative absence of collagen; this process was not detectably altered by either ACTH or Cortisone. Likewise, in the non-scorbutic guinea pigs these hormones were apparently without effect upon wound repair. Morphologic alterations were produced by ACTH and by Cortisone in the adrenal glands. Following ACTH therapy there was elevation of the adrenal/body weight ratio in both scorbutic and non-scorbutic animals, accompanied by histologic evidence of hyperplasia and increased sudanophilia in the zona fasciculata of the adrenal cortex. With Cortisone, on the other hand, in both scorbutic and non-scorbutic guinea pigs the adrenal/body weight ratio was reduced and there was atrophy and storage of lipid in the zona fasciculata. Adrenal concentrations of ascorbic acid were of the same magnitude in all non-scorbutic animals, except for the Cortisone-treated group in which vitamin C levels were uniformly depressed. All animals with scurvy, irrespective of treatment, showed almost negligible amounts of ascorbic acid in the adrenal glands and absence of circulating eosinophils. Eosinophil counts were also reduced by ACTH and Cortisone in the nonscorbutic guinea pigs.

Discussion. It has been suggested that scurvy and the "collagen diseases" have in common an insufficiency of Cortisone-like steroids coupled with continued output of desoxycorticoids(7). This notion is based in part on observations suggesting that Cortisone exerts a beneficial effect in scurvy; yet, paradoxically, the action of this substance on connective tissue has appeared to be inhibitory, or "fibrolytic," in most instances(11-13); however, under certain circumstances fibrosis has been produced(21).

This investigation was undertaken to compare scurvy with hyperadrenalcorticism and to evaluate similarities or differences in their effects upon connective tissue. It was anticipated that scurvy would interfere principally with the formation of collagen. On the other hand, it was thought that the adrenal corticoids would inhibit primarily proliferation of fibroblasts and angioblasts and that the production of ground substance and fibers would be affected only secondarily.

The disturbance of healing manifested by the wounds of our scorbutic animals conforms to that previously described(17,22) and needs no further comment. Surprising, however, was the observation that Cortisone and ACTH, in both scorbutic and non-scorbutic guinea pigs, were without apparent influence upon wound repair. This lack of effect may have been due to inadequate dosage, yet hormone-induced changes were noted in adrenal glands and in eosinophil levels. It remains to be determined whether wound healing might have been affected by larger doses of the hormones or by induction of hyperadrenalcorticism prior to operation. The fact remains that the methods used in this study are essentially like those employed in investigations with other species. It appears, therefore, that in the guinea pig the connective tissue may respond to adrenal hormone, if at all, less readily than in the rat(12,23), the rabbit(11), the mouse(13), and in man(24). These findings also cast doubt on the specificity of the "antiscorbutic" effect of Cortisone reported elsewhere(7-9); such action may represent protection against the non-specific stress of vit. C depletion. This problem clearly demands further study.

The virtual absence of vit. C in the adrenals of all scorbutic animals, despite treatment with ACTH or Cortisone, appears significant. Thus, it may be assumed that the guinea pigs sacrificed on the tenth postoperative day survived at least 5 days with but traces of adrenal ascorbic acid. Furthermore, evidence indicates that adrenal cortical hyperplasia and

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sudanophilia increased during this interval, particularly in the ACTH-treated group, in which the response surpassed that obtained in the corresponding non-scorbutic series. These observations are in accord with those of Hyman, Ragan, and Turner(8), strongly suggesting that vit. C is not essential for the secretion of adrenal hormones. The depletion of adrenal ascorbic acid seen in the non-scorbutic animals receiving Cortisone may be correlated with cortical atrophy and lessened secretory activity; this change was maximal at the end of 10 days. That adrenal concentrations of vit. C were within the normal range following ACTH therapy in the non-scorbutic guinea pigs is consistent with the observed histologic changes and with the duration of the treatment(25).

Our data give no conclusive evidence that either ACTH or Cortisone influenced the rate of growth, nutrition, or hemorrhagic tendency of the scorbutic animals in this study. Definite effects might have been observed had the treatment been prolonged, possibly until spontaneous death. This, however, was deliberately avoided in an effort to secure optimum conditions for appraisal of wound repair.

Summary. 1. ACTH and Cortisone, in doses sufficient to alter the adrenal glands and depress the level of circulating eosinophils, failed to influence the repair of wounds in normal and in scorbutic guinea pigs. 2. It is suggested, therefore, that the connective tissue of the guinea pig may respond to adrenal hormone, if at all, less readily than that of other species studied. 3. The specificity of the "anti-scorbutic" action of Cortisone and of ACTH is questioned. 4. The adrenal response of scorbutic guinea pigs to ACTH, in the virtual absence of adrenal vit. C, suggests that ascorbic acid is not essential for the secretion of adrenal hormone. 5. The relationship of ascorbic acid to the metabolism of the adrenal cortex remains obscure.

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Iodinated Organic Compounds as Contrast Media for Radiographic Diagnoses. X. Interrelationship of Gallbladder and Pancreas.* (18710)

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During a study of the factors influencing the accumulation of cholecystographic media in the gallbladder of dogs, it was found that complete ligation and division of the pancreatic ducts inhibited the visualization of the viscus. This observation indicates that the pancreas plays an intimate role in gallbladder function, and, experimentally, suggests a simple method to test the completeness of ligation and division of pancreatic ducts.

The mechanisms involved in the inhibition of gallbladder visualization after pancreatic duct ligation and division have not been determined as yet. It appears that the effect requires several days for development since dogs with pancreatic duct ligation, or with complete pancreatectomy, visualize satisfactorily if intravenous cholecystography is carried out immediately after the completion of the operative procedure. Provisionally, the inhibition of visualization seems to be due to some alteration in the concentrating mechanism of the gallbladder.

Experimental. Studies were carried out on 14 healthy, mongrel dogs ranging in weight from 13.5 to 25 kg. Preoperative control cholecystograms were made, usually by intravenous administration of the sodium salt of

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TABLE I. Quality of Gallbladder Visualization Following Ligation and Division of Pancreatic Ducts in Dogs that Visualized Well Preoperatively.

Dog	Days post-operative	Route of administration	Medium	Gallbladder shadow	Autopsy findings
1609	8	Oral	II	0	Extensive atrophy of pancreas with fibrosis; no evidence of reestablishment of ductal system.
	12	Intrav.	II	0	
	23	”	II	Faint	
1608	7	”	II	0	All but 3 cm of pancreas atrophied and fibrosed; gallbladder dilated and contains black cryst. material.
	10	”	II	0	
	17	”	III	0	
	22	”	II	Faint	
1680	6	Intrav.	II	Faint	Pancreas atrophied to a considerable degree; pancreatic ducts dilated. Extra-hepatic biliary tract dilated — G.B. cont. thick viscus bile.
	14	Oral	II	”	
	36	Intrav.	II	Fair	
1684	6	”	II	0	Pancreas atrophied with normal tail section; gallbladder dilated.
	30	”	II	Faint	
	34	”	II	”	
	38	”	II	”	

a cholecystographic medium. The media used were: β-(4-hydroxy-3,5-diiodophenyl)-α-phenylpropionic acid (I), o-(4-hydroxy-3,5-diiodobenzyl)-benzoic acid (II), and phenoltetraiodophthalein (III)(1). The level of dosage was constant for a given dog, and 50 mg/kg was satisfactory for most of the studies. The radiographs were made under barbiturate anesthesia eighteen hours after oral and 2 or more hours after intravenous administration. The general procedure for radiography is described in an earlier publication(1). The dogs were operated under sterile conditions. The ducts were isolated with a minimum of trauma to the gland, doubly tied with fine silk, and cut; the duodenal stump of the pancreatic duct was inverted. In later operations, to ensure that all branches of the ductal system were located, the gland was separated from the duodenum, and care taken not to interfere with the blood supply. Following a recovery period of 6-8 days, cholecystographic media were administered to the dogs at varying intervals. The same medium was used for the terminal radiographic study as for the initial control examination. Finally, the dogs were sacrificed, and the pancreas, liver, and the extra-hepatic biliary tract examined grossly and microscopically.

Results. In 7 of the 14 operated dogs, gallbladder visualization was inhibited after the operative procedure. The faint shadows that were obtained revealed that the viscus was greatly dilated in 6 of the 7 animals. At autopsy the dogs showed atrophy of the acinar tissue, varying degrees of fibrosis, greatly dilated pancreatic ducts, and dilated gallbladders. Stones were found in one dog, and a black sludge in the bile of 2 other animals. Representative data from the experiments are collected in Table I.

In 5 operated dogs the gallbladder visualized satisfactorily. At terminal laparotomy or autopsy, incomplete ligation or regeneration of ducts was evidenced by the presence of normal appearing pancreatic tissue with patent ducts communicating with the duodenum. In 2 animals the patency of the ducts was demonstrated by the free flow of pancreatic juice following secretin stimulation.

That the effect is not one that develops immediately is shown by the results obtained with 2 dogs that were used for acute experiments. Intravenous cholecystography with medium II was carried out immediately after the pancreatic ducts had been tied and cut in one animal, and shortly after complete pancreatectomy in the other dog. The gallbladders of both dogs visualized well after a 2 hour interval.

Discussion. The experimental results dem-

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onstrate the close relationship of the gallbladder and the pancreas in a novel way, and suggest that the pancreas controls the tone of the gallbladder. The operative procedure deprives the duodenum of the external secretion of the pancreas and at the same time leads to atrophy of the organ. It is conceivable that lack of sodium bicarbonate and of the pancreatic enzymes in the duodenum may bring about an alteration in the hormonal control of the gallbladder. On the other hand, atrophy of the pancreas may lead to the deficiency of some principle from the pancreas that is essential to good gallbladder function.

After ligation and division, the pancreatic ducts have a marked tendency to recanalize or to establish collateral ducts. Since this occurred in 5 of the dogs, these processes evidently take place fairly readily. This group of unsuccessful operations provides evidence, however, that surgical manipulation of itself is not responsible for the failure of gallbladder visualization in the successfully operated animals. Presumably, the surgical trauma and degree of innervation to the gallbladder region is the same in both groups of animals.

As this work shows, there is always some uncertainty as to the operative result when the pancreatic ducts are tied and cut. Use of intravenous cholecystography, particularly with *o*-(4-hydroxy-3,5-diiodobenzyl)-benzoic acid, appears to furnish a convenient way of establishing the status of the pancreas and the pancreatic ducts in operated dogs. Additional work will be required to determine the limitations of the method and whether it is valid in dogs after an extended period of time.

In some ways the experimental results parallel certain clinical observations. Elman(2) pointed out that the gallbladder may not visualize if intravenous cholecystography is carried out during episodes of acute pancreatitis. Since then this observation has been confirmed by Silvani and McCorkle(3). Comfort and associates(4) have added further clinical reports on the failure of the gallbladder to visualize during acute and chronic pancreatitis. It is evident from the several reports, however, that the clinical picture is not too well defined.

Additional work is in progress to clarify both the experimental and clinical aspects of the interrelationship of the pancreas and the gallbladder.

Summary. Studies on dogs show that successful ligation and division of the pancreatic ducts inhibits the visualization of the gallbladder by cholecystographic media whether administered orally or intravenously. Failure of visualization appears to be due to the development of physiological stasis of the viscus. The experiments demonstrate a close relationship of the two organs and suggest that the pancreas may control the tone of the gallbladder

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Depression of Fecal Urobilinogen by Aureomycin and by Terramycin.* (18711)

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Modification of the intestinal flora by drugs and the resultant effects on the nutrition and

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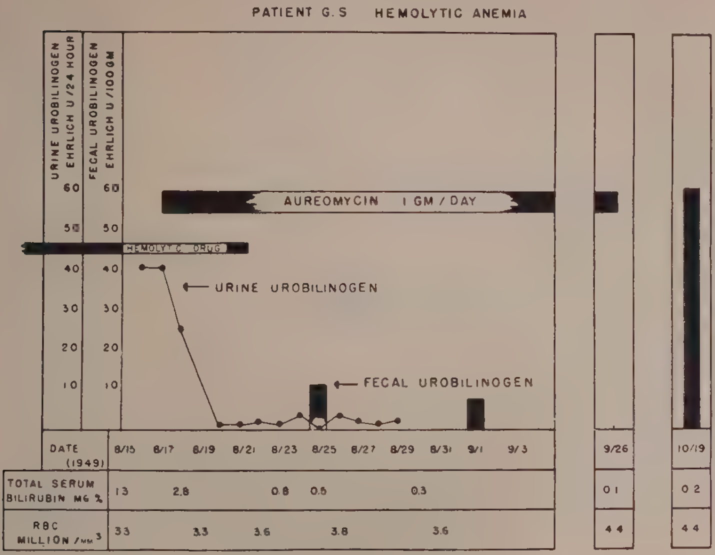

FIG. 1.

Changes in urinary and fecal urobilinogen concentration in a patient with hemolytic anemia who received aureomycin. Fecal urobilinogen concentration is indicated by the vertical bars.

metabolism of the host have received extensive study in experimental animals(1,2). It has been observed in man, also, that the administration of certain antimicrobial agents causes a decided alteration in the bacterial pattern of the intestinal contents(3).

It has been reported that the administration of aureomycin results in a marked reduction of urobilinogen in the feces of man, and that this change takes place concurrently with a reduction in the number of coliform bacteria in the feces(3c,d). It is the purpose of the present report to describe similar observations made in this laboratory on the fecal urobilinogen concentration of subjects who received aureomycin and terramycin.

Subjects and procedures. Patient G.S. was a 28-year-old white female with far advanced pulmonary tuberculosis and neuromyelitis optica who developed a hemolytic syndrome as the result of the administration of an experimental drug. Prior to aureomycin therapy the patient presented definite evidence of moderately severe hemolysis. The erythrocyte count was 3.3 million per cu mm, the serum bilirubin 1.3 mg per 100 ml, and the urine urobilinogen 40.5 Ehrlich units per 24 hours. Because of an intercurrent urinary tract in-

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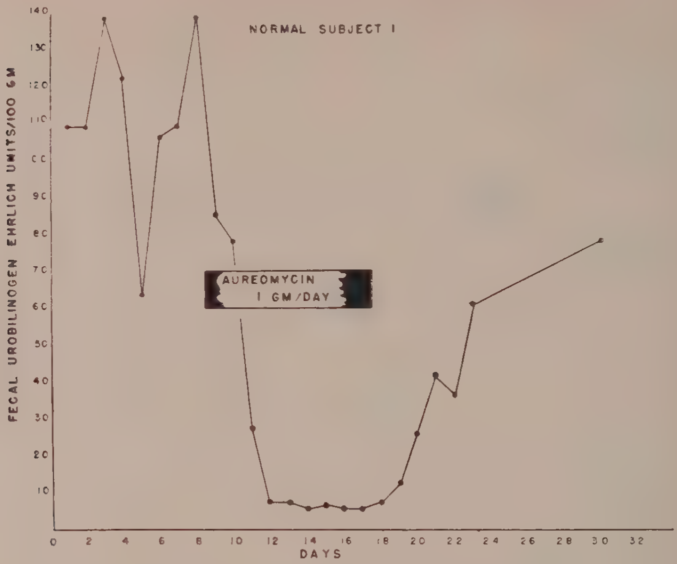

FIG. 2.

The fecal urobilinogen concentration of a normal subject who received aureomycin. Values are representative of those observed in 3 subjects.

fection the patient received aureomycin orally, 0.25 g every 6 hours, for 39 days (Fig. 1). Observations on the effect of aureomycin on fecal urobilinogen concentration were made in 3 normal persons. One subject received 1 g of aureomycin a day in divided doses for 9 days; one received 2 g a day for 5 days; and one received 2 g a day for 2 days. Observations on the effect of terramycin were made on stools from 7 patients receiving terramycin because of respiratory and urinary tract infections, but who were otherwise normal. Urine and fecal urobilinogen concentrations were measured by the method of Watson *et al.* (4) employing a Coleman junior spectrophotometer calibrated with Pontacyl dye standard. A wave length setting of 565 mμ was used. Twenty-four-hour urine volumes were measured on patient G.S. and urine urobilinogen excretion expressed in Ehrlich units per 24 hours (normal values range from 1 to 8 Ehrlich units per 24 hours). Fecal urobilinogen concentration was expressed in Ehrlich units per 100 g wet weight (normal values range from 40 to 160 Ehrlich units per 100 g). Urine specimens were kept stoppered and in the dark until tested. Stool specimens were stored in the refrigerator and were tested within 24 hours after collection.

Results. The observations made on the patient with hemolytic anemia (G.S.) are presented in Fig. 1. The urinary urobilinogen, decidedly elevated as a result of hemolysis, fell during the 72 hours following the initiation of aureomycin therapy to levels below 1 Ehrlich unit per 24 hours. It remained in this range, not exceeding 3 Ehrlich units per 24 hours, during the subsequent 9 days of aureomycin therapy during which measurements were made. Stool urobilinogen concentrations were measured on the 8th and 15th days of aureomycin therapy and were very low, being 13 and 8 Ehrlich units per 100 g, respectively. Although this patient's fecal urobilinogen concentration was not measured prior to aureomycin therapy, it is very probable that it was well above normal, in view of the frank hemolytic syndrome which she presented. Three weeks following the discontinuance of aureomycin, the patient's

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fecal urobilinogen concentration was normal.

The oral administration of aureomycin to the 3 normal subjects resulted, in each case, in a fall in stool urobilinogen to extremely low levels within 48 to 72 hours. The data presented in Fig. 2 are representative of the changes observed in all subjects. In all 3 subjects the range of fecal urobilinogen concentration after 48 to 72 hours of aureomycin administration was 4 to 8 Ehrlich units per 100 g.‡ One subject ingested the drug for only 48 hours, but this was sufficient to result in a sharp fall in the fecal urobilinogen from 100 to 6 Ehrlich units per 100 g.

After the discontinuance of aureomycin there was, in each subject, a period of 3 to 6 days during which pre-treatment fecal urobilinogen concentrations were gradually reestablished (Fig. 2). In no case was a normal concentration attained prior to 3 days after aureomycin.

The administration of terramycin was found, in a group of 7 patients, to produce a depression of fecal urobilinogen similar in all respects to that produced by aureomycin.

Discussion. In the present study it was observed that the urobilinogen content of the feces of a person ingesting aureomycin or terramycin diminished to a barely measurable concentration during the first 48 to 72 hours. It was maintained at this level throughout the duration of the treatment period, at least for as long as 14 days. Pre-treatment urobilinogen concentrations were usually restored within 6 days following the discontinuance of the drug.

The dynamics of this change in fecal urobilinogen content strongly suggest that it is the result of a distinct alteration in the intestinal flora. It is well known that other more obvious changes in the feces, indicative of altered flora, occur during aureomycin therapy. The stool becomes less solid, practically odorless, may change in color from a brown to a dark green, and sometimes has been observed to increase in bulk. The change in color may be reasonably explained as being due to the accumulation of bilirubin and its green oxidized derivative, biliverdin, due to the failure of the intestinal microorganisms to reduce bilirubin. The loss of the characteristic fecal odor apparently reflects the suppression of the putrefactive organisms in the intestine, which are capable of splitting proteins and of producing indole, skatole, and other amines responsible for fecal odor. The organisms believed most important in these putrefactive processes are the anaerobic bacilli and *E. coli*(7a).

The exact alterations in intestinal flora by which aureomycin or terramycin results in depression of the fecal urobilinogen have not been completely ascertained. Unfortunately, little is known regarding the precise nature of the organisms which reduce bilirubin to urobilinogen in the normal intestine. Kammerer and Miller (5) studied by *in vitro* methods, many of the organisms which normally inhabit the intestine and found that none, including *E. coli* and the obligate anaerobic spore-forming bacillus *B. putrificus,* was capable in pure culture of reducing bilirubin to urobilinogen. However, when bile was added to a mixed culture of *B. putrificus* and *E. coli,* the reduction was accomplished. Several aerobic species other than *E. coli* were capable of complementing the action of *B. putrificus* in this process. The presence of the anaerobic bacillus, however, was found essential to the reduction. These investigators concluded, therefore, that the reduction of bilirubin to urobilinogen *in vitro* was the result of the synergistic action of the obligate anaerobic bacillus, *B. putrificus,* and aerobic bacteria such as members of the coliform group. Furthermore, Kammerer and Miller linked the reduction of bilirubin with the formation of indole and the putrefactive processes, which are believed to be the result of the strong reducing properties of the anaerobes of the intestine(6).

Preliminary observations on the quantitative changes in the fecal flora during aureomycin or terramycin therapy made elsewhere

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‡ These values represent density readings below .05 on the Coleman junior spectrophotometer. Because of the large error involved in readings in this range, they do not necessarily indicate the presence of any urobilinogen at all.

5. Kammerer, H., and Miller, K., *Deut. Arch. f. Klin. Med.,* 1923, v141, 318.

(3c,e-h), have indicated the most striking change in the *aerobic* portion of the flora to be a sharp decrease in the number of coliform bacilli concomitant with an increase in *B. proteus*. Apparently this is not the only change in bacterial pattern, however, as the loss of fecal odor would indicate the depression of those organisms which form odoriferous amines, *i.e.* the anaerobic bacilli.

On the basis of the present state of knowledge it would appear that the most acceptable hypothesis as to the mechanism of depression of fecal urobilinogen by antimicrobial agents, such as aureomycin or terramycin, is that it results from the elimination of, or marked reduction of, both the coliform bacilli and the putrefactive anaerobic bacilli in the bowel.

The practical clinical importance of the fact that some antimicrobial drugs depress fecal urobilinogen lies in the diagnosis of hemolytic anemias and the cause of jaundice. It is apparent that misleadingly low values would result in cases of hemolytic anemia, as in the case of patient G.S.; and in other cases of jaundice. Measurements of fecal urobilinogen are only truly representative of the amount of bile pigment in the intestine if the patient has not ingested aureomycin, or a similar antibacterial compound, within the preceding 6 or 7 days.

Summary. The oral administration of aureomycin or terramycin to man results in an extremely low concentration of fecal urobilinogen; a change which occurs during the first 48 to 72 hours of drug therapy, concurrently with other modifications in the character of the intestinal contents. Normal concentrations of urobilinogen may not be restored for so long as six or seven days following the discontinuance of drug administration. The depression of urobilinogen formation is probably the result of alterations in the flora of the intestine, which have not yet been completely defined. A case of hemolytic anemia is reported in which strikingly low fecal and urinary urobilinogen concentrations were observed while the patient was receiving aureomycin.

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6. (a) Hawk, P. B., and Bergeim, O., *Practical Physiological Chemistry*. Blakiston, 11th Ed., 1937. Chap. XX. (b) Dakin, H. D., *Oxidation and Reduction in the Animal Body*. Longmans, Green and Co., 1922, New York.

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In vitro and *in vivo* Studies of a New Antibiotic, Fumagillin, with *Endamoeba histolytica*.* (18712)

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(Introduced by Hamilton H. Anderson)

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Many antibiotics have been found to be effective both *in vitro* and *in vivo* against *Endamoeba histolytica*. For *in vitro* studies a culture of *E. histolytica* particularly appropriate for the determination of "direct amebacidal" action by agents which do not exert any effect on organisms growing in association with the ameba has recently been developed(1,2). A new antibiotic, designated "Antibiotic H-3" or fumagillin, has been kindly supplied to us for experimental trial by the Upjohn Co. and by Eli Lilly and Co. Fumagillin was first isolated from *Aspergillus fumigatus* by Eble and Hanson(3,4) in the Research Laboratories of The Upjohn Co.,

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* Supported in part by the National Institutes of Health, Bethesda, Md., and the Abbott Laboratories, North Chicago, Ill.

† With assistance of Miss Dorothy Swanman.

1. Phillips, B. P., and Rees, C. W., *Am. J. Trop. Med.*, 1950, v30, 185.

2. Phillips, B. P., *Science*, 1950, v111, 8.

and McCowen, *et al.*(5) of the Lilly Laboratories were the first investigators to demonstrate its highly effective amebacidal properties *in vivo* and *in vitro*.

This paper is a report of studies undertaken to determine the *in vitro* effectiveness of this new antibiotic with some preliminary observations relative to the treatment of macaques infected with *E. histolytica*. Our investigations were based primarily on the initial studies by McCowen and his associates(5).

Materials and methods. The "amebacidal" end-point of fumagillin was determined by the procedure described in our earlier work (6,7). The culture of *E. histolytica* (F-22) associated with *Trypanosoma cruzi* (Culbertson) was obtained from Dr. C. W. Rees, National Institutes of Health, Bethesda, Md.(1). Since this antibiotic was found to be insoluble in water, 0.01 N NaOH was utilized as a solvent. The alkalinized solution of fumagillin was tested in serial dilutions against a 48-hour culture of *E. histolytica* growing in association with *T. cruzi*. The culture tubes were incubated at 37°C for 48 hours and examined. Subcultures were made from all the tubes in which amoebas were not demonstrated. To study the effect of this antibiotic on the trypanosomes, the "amebacidal" concentrations were tested against the trypanosomes alone. To determine the toxicity of the solvent 0.01 N NaOH, against the amoeba, controls were run with the alkali and no antibiotic. Chemotherapeutic studies were undertaken with two species of monkeys, *Macacus rhesus* and *M. philippinensis* naturally infected with *E. histolytica*. The majority of the animals had diarrheic stools before therapy. Their original weights varied from 1.5 to 4 kg. The monkeys were segregated in individual cages which were cleaned and scrubbed with lysol daily. The excreta dropped through wire floors on the bottom of the cages beyond the reach of the animals. Stool specimens were collected in the morning on clean paper towels placed on receiving pans under the wire floors. Fecal smears made promptly within 30 minutes after passage of the stools were fixed in Schaudinn's fluid. For food the monkeys were given cooked beans or wheat enriched by powdered milk, dry yeast and liver fraction L (Wilson Laboratories). In addition they received oranges daily and dog biscuits 2 or 3 times a week. The animals were irradiated with ultra-violet light for 1 to 2 hours daily. The fecal smears were stained by Heidenhain's iron-hematoxylin method. During the follow-up examination period after therapy 2 smears from each monkey were searched for protozoan parasites on 5 consecutive days every 2 weeks.

Results and discussion. The "amebacidal" level of this new antibiotic was found to be in dilutions from 1:10,000,000 to 1:15,000,000. The trypanosomes were not affected by these concentrations of the antibiotic. 0.01 N NaOH used as a solvent for the antibiotic was not toxic for the ameba. Fumagillin, or "Antibiotic H-3", was found to exert the most active *in vitro* effect of all the numerous antibiotics and other agents which have been tested in this laboratory against *E. histolytica*(6-9).

Fourteen monkeys were treated with fumagillin, each animal receiving from 50 to 125 mg/kg of the drug by mouth daily for 5 days. The results are presented in Table I. These preliminary therapeutic studies indicate that this new antibiotic is effective against *E. histolytica*. Follow-up examinations have disclosed that 4 of the 14 animals have shown *E. histolytica* in their stools following treatment with fumagillin. Monkeys No. 2, No. 3, No. 8 and No. 9 were found to be clear of amoebas at the end of 14 weeks. Monkey No. 6 died of tuberculosis at the end of one month

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3. Hanson, F. R., and Eble, T. E., *J. Bact.*, 1949, v58, 527.

4. Eble, T. E., and Hanson, F. R., *Antibiotics and Chemotherapeutics*, in press.

5. McCowen, Max C., Callender, M. E., and Lawlis, John F., Jr., *Science*, 1951, v113, 202.

6. Nakamura, M., and Anderson, H. H., *J. Pharm. and Exp. Therap.*, in press.

7. Nakamura, M., and Anderson, H. H., *Exp. Parasitol.*, in press.

8. Bradin, J. L., and Hansen, E. L., *Am. J. Trop. Med.*, 1950, v30, 27.

9. Anderson, H. H., and Anderson, J. V. D., *Am. J. Trop. Med.*, 1950, v30, 193.

TABLE I. Effect of Fumagillin Administered Orally on *Endamoeba histolytica* Occurring Naturally in Macaques.

Macacus rhesus or *M. philippinensis*	Dose of fumagillin, mg/kg	Duration of treatment, days	Recurrence of *E. histolytica* in stools after treatment
2	50	5	No recurrence after 14 wks
3	50	5	" " 14 "
6	50	5	" " 4 " Animal died of TB.
8	100	5	No recurrence after 14 wks
9	100	5	" " 14 "
S	100	5	Recurrence in 12 wks
M	100	5	" 10 "
11	100	5	" 10 "
R	100	5	" 4 "
R_1	125	5	No recurrence after 8 wks
S_1	125	5	" " 8 "
M_1	125	5	" " 8 "
10	125	5	" " 8 "
11_1	125	5	" " 8 "

following therapy; however, during this post-treatment period there was no recurrence of *E. histolytica* in the stools. Monkeys R_1, S_1, M_1, 10 and 11_1 showed no recurrence of *E. histolytica* at the end of 8 weeks.

The effect of fumagillin on non-pathogenic amebas could not be evaluated since only 3 monkeys had such amebas in their stools before the treatment for *E. histolytica* was instituted. However, it was found that 2 of these monkeys were cleared of *Endamoeba coli* during a 14 weeks' follow-up examination period. No toxic manifestations were observed in any of the monkeys treated with the antibiotic. The amount of hemoglobin and the number of erythrocytes and leucocytes remained within normal limits. Bromsulfalein tests indicated a possibility that a dose of 125 mg/kg might cause liver damage. In monkey S there was considerable retention of the dye. This animal has periodic bromsulfalein tests at the present time. Blood urea nitrogen showed an increase in only one monkey; this animal had received 100 mg of fumagillin per kilo of body weight. Electrocardiograms of all the animals were normal following therapy.

Summary. 1. A new antibiotic, fumagillin ("Antibiotic H-3"), was found to be effective *in vitro* against *E. histolytica* at concentrations of 1:10,000,000 to 1:15,000,000. 2. Preliminary *in vivo* studies on monkeys naturally infected with *E. histolytica* indicated that fumagillin possesses considerable amebacidal activity. Further investigations are necessary to evaluate this drug more completely.

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Effect of Sodium Bisulfite and Rutin on Action of Epinephrine. The "Bisulfite Phenomenon" in Man.* (18713)

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Richards(1) reported that sodium bisulfite increased the toxicity of epinephrine if these two compounds were injected subcutaneously or intramuscularly at the same site, but when given intravenously or injected at different sites no increase in toxicity, as measured by lethal doses, was noted. A review of the literature(2) as well as Richards' own studies showed that sodium bisulfite itself is relatively non-toxic. The U.S.P. permits the use of sodium bisulfite in concentrations up to 0.5% as an antioxidant in commercial epinephrine solutions, although not more than 0.2%

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* This work was aided by a grant from Abbott Laboratories.

† Jessie Horton Koessler Fellow.

1. Richards, R. K., *J. Pharm. and Exp. Therap.*, 1943, v79, 111.

2. Heffter, A., *Handbuch der experimentellen Pharmakologie*, 3: 1. Berlin, 1927.

is generally used in the 1:1,000 epinephrine hydrochloride solutions. Richards found that not only does sodium bisulfite enhance the subcutaneous and intramuscular toxicities of epinephrine hydrochloride solutions in rats, mice, rabbits and dogs but that non-toxic doses increase the epinephrine effects on the blood sugar curve of dogs and also have an effect on the action of other drugs (neosynephrin(3) and procaine)(4). To show that sodium bisulfite is specific, Richards(1) also tried other drugs and other antioxidants (HCl, ascorbic acid, hypophosphorous acid, sodium hypophosphite, sodium acid phosphate) and these did not influence the resorptive toxicity of the epinephrine solution. As all the previous work has been done on animals we sought to determine if this "bisulfite phenomenon", which presumably acts by increasing the rate of absorption of epinephrine, also occurs in man when ordinary therapeutic doses are used.

Richards and Kueter(5) showed that the increase in epinephrine toxicity in rats caused by sodium bisulfite was abolished by previous administration of rutin, whereas the toxicity of epinephrine solution alone was not affected by rutin. We also tested the effect of rutin on the "bisulfite phenomenon" in man.

Method and results. 1. *Action of epinephrine solution using sodium hypophosphite as a preservative.* (a) In 19 male subjects blood pressure and pulse rate determinations were made every 10 minutes for 20 minutes before, and one hour after injection of the drug. One cc of a 1 to 1,000 solution of epinephrine with 0.5% sodium hypophosphite was injected subcutaneously. The systolic blood pressure rose slowly having risen an average of 9.0 mm of Hg at the end of one hour. The diastolic pressure fell an average of 17.2 mm of Hg and the pulse rate increased 8.6 beats per minute (Refer to Fig. 1). (b) In order to follow the blood pressure until it returned to normal and to determine the time of the peak of action, the experiments were repeated on six subjects and the blood pressure and pulse rates were followed until they returned to control levels. These results showed that the systolic pressure peak occurred on the average of 56.7 minutes after injection (Refer to Fig. 2). (c) The same procedure was followed on two subjects using epinephrine powder freshly put into solution with no preservative added and the curve of response was not significantly different from

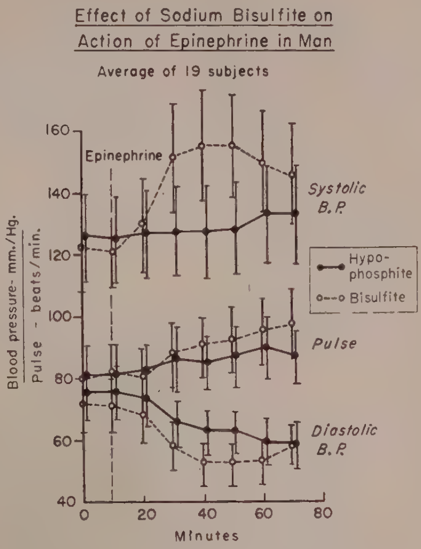

FIG. 1.

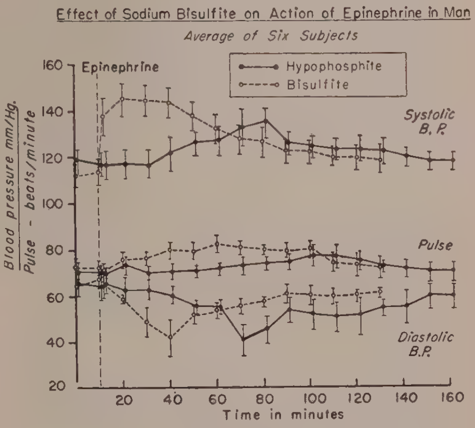

FIG. 2.

The vertical bars represent the standard error of the means.

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3. Richards, R. K., *Fed. Proc.*, 1943, v2, 89.

4. Richards, R. K., and Kueter, K. E., *Anesth. and Analg.*, 1943, v22, 283.

5. Richards, R. K., and Kueter, K. E., *J. Pharm. and Exp. Therap.*, 1948, v94, 372.

TABLE I. Effect of Sodium Bisulfite and Rutin on the Action of Epinephrine Average of 6 Subjects.

	Without rutin				With rutin	
	Absolute change from control level		Difference		Absolute change from control level	
	Hypophosphite	Bisulfite	Avg	t	Hypophosphite	Bisulfite
Systolic B/P—mm Hg	20.7	41.7	21.0	5.68	24.4	42.6
Diastolic B/P ”	–28.0	–28.0	0	—	–28.0	–28.6
Systolic B/P peak—min.	56.7	14.3	42.4	3.34	26.7	21.3
Diastolic B.P. ” ”	68.3	30.0	38.3	3.9	68.3	31.8
Pulse—beats/min.	11.0	14.0	3.0	0.26	12.4	12.0

that obtained when sodium hypophosphite was used.

2. *Action of epinephrine solution using sodium bisulfite as a preservative.* (a) The same procedure was followed as in 1 (a) above on the same 19 subjects with the exception that 0.1% sodium bisulfite was used as the antioxidant for the epinephrine. (Refer to Fig. 1.) The systolic blood pressure rose an average of 34.7 mm of Hg, the diastolic pressure fell 19 mm of Hg and the pulse rate increased 15 beats per minute. (b) The same procedure was followed on the same 6 subjects as in 1 (b) above except that sodium bisulfite was used in place of the sodium hypophosphite. The systolic pressure peak occurred in 14.3 minutes (refer to Fig. 2). These results show that the absolute increase in systolic pressure in response to epinephrine solution with sodium bisulfite was 21.0 mm of Hg higher than the sodium hypophosphite (t = 5.68); and the time of peak of action was 42.4 minutes sooner (t = 3.34). The diastolic pressures were decreased with both drugs to the same degree (–28 mm Hg). The difference in the pulse rate was not statistically significant (t = 0.26) (Refer to Table I).

3. *Action of epinephrine solution after the injection of rutin.* (a) Using sodium hypophosphite as a preservative the same procedure was followed on the same 6 subjects as in 1 (b) above with the exception that 125 mg of rutin (solubilized with piperazine) in 20 cc of saline was given slowly intravenously over a period of 4 minutes before the epinephrine with sodium hypophosphite was given. The blood pressure and pulse rates taken directly after the rutin showed no change and the epinephrine was injected immediately therafter. The systolic pressure rose an average of 24.4 mm of Hg with the peak occurring in 26.7 minutes. The diastolic pressure fell 28 mm of Hg and the pulse rate increased 12.4 beats per minute. (b) Using sodium bisulfite as a preservative the same procedure with rutin was followed on the same 6 subjects as in (a) above with the exception that sodium bisulfite was used as the preservative for the epinephrine solution. The systolic blood pressure rose an average of 42.6 mm of Hg with the peak occurring in 21.3 minutes. The diastolic pressure fell 28.6 mm of Hg and the pulse rate increased 12 beats per minute. These results with the rutin are not significantly different from those without the rutin.

Richards(3) found that a dose of about 350 mg per kg of rutin was required to counteract the "bisulfite effect" in rats. Since our dose in human subjects was only about 2 mg per kg, it is not surprising that the rutin failed to alter significantly the "bisulfite effect".

A number of studies including a recent one by Crismon and co-workers(6) have shown that intravenously injected rutin potentiates the action of topically applied epinephrine on the vessels of the rat's mesoappendix. Crismon *et al.*(6) found a dose of 2.5 mg/kg adequate to produce this action. It is interesting to note that in the present study doses of 2 mg/kg given intravenously in human subjects failed to alter the action of epinephrine on blood pressure. In studies on anesthetized dogs we have found that rutin in doses as

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6. Crismon, J. M., Berez, R. R., Madden, J. R., and Furhman, F. D., *Am. J. Physiol.*, 1951, v164, 391.

high as 50 mg/kg given intravenously does not alter the pressor response to small doses of epinephrine.

Summary and conclusions. The subcutaneous injection of epinephrine solution containing sodium bisulfite as an antioxidant produced a more rapid and higher rise in systolic blood pressure than did the same amount of epinephrine in a solution containing sodium hypophosphite as a preservative. Small doses of rutin given intravenously immediately before the epinephrine did not significantly alter the "bisulfite effect" nor did the rutin influence the effect of epinephrine.

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Leukocyte Response to Stress in Normal and Adrenalectomized Rats Pretreated with Ascorbic Acid. (18714)

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Ascorbic acid treatment to animals exposed to stress has been observed to exert certain protective actions which are related to adrenal cortical function(1-3). This ability of ascorbic acid has been established by survival studies, and by studies on the functional status of the cortex. It was demonstrated that ascorbic acid pretreatment to intact rats prevented the eosinopenia following epinephrin stress; this was associated with a prevention of the depletion of adrenal steroids(3).

This paper reports results which indicate that there may exist an ascorbic acid-eosinophil alliance independent of the adrenal.

Materials and methods. Adult female Osborne-Mendel rats were used in this study. Twelve sham-adrenalectomized rats (7-8 days post-operative) were divided into 2 groups. Group I animals were pretreated with ascorbic acid (Vitamin C injectable, sodium ascorbate, Roche), 87.5 mg/100 g in two intra-peritoneal injections; Group II rats received pretreatment with saline solution. Two groups of adrenalectomized rats, (7-8 days post-operative) were given respectively, pretreatment with ascorbic acid, (Group III), and pretreatment with saline (Group IV). About 6-8 hours after the last pretreatment injection, all animals were given single subcutaneous injections of epinephrin at the rate of 0.03 mg/100 g. Total and differential leukocyte counts (Randolph's method)(4) of the tail blood were made of all animals in the following instances: Count (a) before pretreatment, Count (b) 3 hours after the injection of epinephrin, and Count (c) 24 hours after epinephrin. The mean differences of Counts (b), and (c) from Count (a) were calculated and are presented along with the standard errors in Table I. Counts made previous to the experiment indicated that the operation of sham-adrenalectomy did not disturb the leukocyte picture, but adrenalectomy led to a significant leukopenia, lymphopenia, and eosinopenia. Adrenalectomy did not however, affect the polymorphonuclear count.

Results. Both groups of sham-adrenalectomized rats (I and II) exhibited a significant decrease of lymphocytes 3 hours after the injection of epinephrin and returned to prestress levels 24 hours after the stress. The injection of epinephrin to adrenalectomized rats (III and IV) failed to alter the number of circulating lymphocytes. The expected rise in polymorphonuclear leukocytes (neu-

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1. Dugal, L. P., and Therien, M., *Endocrinology*, 1949, v44, 420.

2. Dugal, L. P., and Leblanc, J., *Rev. canad. de biol.*, 1949, v8, 440.

3. Bacchus, H., and Toompas, C. A., *Science*, 1951, v113, 269, 367.

4. Randolph, T. G., *J. Allergy*, 1944, v15, 89.

TABLE I. Leukocyte Response to Stress.

		Mean Differences (M.D.) from control levels.*							
	Type of rat	Sham-adrenalectomized‡				Adrenalectomized‡			
	No. rats	6		6		6		6	
	Pretreatment†	Saline		Ascorbic acid		Saline		Ascorbic acid	
	Hr after epinephrin	3 (a-b)*	24 (a-c)	3 (a-b)	24 (a-c)	3 (a-b)	24 (a-c)	3 (a-b)	24 (a-c)
Change of the leukocytes	Leukocytes	+2.5	-.8	-.5	-1.5	+5.4§	-.5	+8.6§	-.2
	1000/mm³ ± S.E.	2.3	.5	1.4	1.8	.9	1.0	3.5	.9
	Lymphocytes	-2.5§	-.5	-3.3§	-.6	+.1	+.2	+6.9	+.5
	1000/mm³ ± S.E.	.9	.3	1.3	1.1	.5	1.1	3.6	1.1
	Polymorphs	+4.9§	-.3	3.4§	-.8	+5.3§	-.9	+3.5§	-.6
	1000/mm³ ± S.E.	1.3	.2	.9	.3	.7	.5	1.1	.3
	Eosinophils	-176§	+22	+242§	+132	+484§	+110	+176	+506§
	per mm³ ± S.E.	24	36	88	66	95	45	97	96

* Count (a) was made just prior to pretreatment; count (b), 3 hr following epinephrin; count (c), 24 hr after epinephrin. (a-b) = count (a) compared to count (b).

† Pretreatment with saline or with ascorbic acid 7 to 8 days after operation.

‡ 7-8 days post-operative.

§ Mean difference is significant.

trophils) occurred in sham-adrenalectomized rats 3 hours after, and returned to pre-stress levels 24 hours after, the epinephrin injection. A significant rise in the neutrophil count was observed in all adrenalectomized rats (III and IV) 3 hours after the stress, these returned to pre-stress levels by 24 hours following the injection.

The eosinophil response. The expected eosinopenia 3 hours after stress was observed in Group II rats but was absent in animals pretreated with ascorbic acid (Group I). There was a significant rise in the number of circulating eosinophils in the Group I rats; this rise of eosinophils was similar to the rise observed in adrenalectomzed rats 3 hours after epinephrin, and was observed in a previous study in intact rats(3). In the rats which were adrenalectomized and supplied with ascorbic acid (Group III), the rise of eosinophils was delayed to 24 hours after the injection of epinephrin; it is noted that within this same period the levels in Group II and Group IV rats returned to prestress levels.

Discussion. The data presented in Table I show the response of the blood leukocytes to stress under a variety of conditions. Since the response was of importance, only the mean differences and the standard errors are presented in the table, the absolute numbers are not presented. The data suggest that ascorbic acid may exert an effect on the leukocyte picture even in the absence of the adrenal. It is noteworthy that the eosinophil response of the ascorbic acid pretreated sham-adrenalectomized rat is similar to that of the adrenalectomized rat exposed to stress. This observation is in consonance with observations that the vitamin prevented the adrenal hypertrophy following stress, and the depletion of steroids characteristic of stress(2,3). It is also apparent that ascorbic acid pretreatment to adrenalectomized rats altered the response of the eosinophils to epinephrin stressor. Instead of the rise observed in this study in pretreated animals, and in other studies(5), at the end of 3 hours after epinephrin, there is a delay of the response. Whether this delay is of any significance remains to be determined but it is very evident from these data that ascorbic acid modified the response of the eosinophils in the absence of the adrenal gland. It is well known that the leukocytes are concerned with the transportation of ascorbic acid(6). Should the action of the vitamin observed in this study involve any action on the transportation of ascorbic acid, then it becomes necessary to assume that the eosinophils and not the other elements of the leukocytes are probably involved in these changes. It has been suggested that ascorbic acid is related to the production and utilization of

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5. Dury, A., *Am. J. Physiol.*, 1950, v160, 75.
6. Heinemann, M., *J. Clin. Invest.*, 1941, v20, 467.

adrenal cortical hormones(7,8), but it is not clear whether these possible functions of ascorbic acid are related to its alliance with the eosinophils.

Summary. The action of ascorbic acid on the leukocyte response of normal (sham-adrenalectomized) and adrenalectomized rats was investigated. The data indicate that, of all the leukocytes studied, only the eosinophils are related to the action of ascorbic acid. The vitamin alters the eosinopenia of stress in intact rats, and delays the eosinophilia characteristic of stress in adrenalectomized rats.

The author acknowledges the helpful interest of Doctor C. E. Leese.

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8. Giroud, A., and Martinet, M., *Comp. rend. soc. biol.*, 1941, v135, 1344.

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A Continuously Recording Scanning Device for Radio-Chromatographic Analysis of Labelled Antigen-Antibody Reactions.*† (18715)

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This communication describes an application of paper chromatography to the study of interactions of antigen with antibody and a method of continuous recording to indicate the distribution of radio-isotopically labelled components. Castaneda(1) has described some reactions of Brucella antigens with their antisera on paper chromatograms using hematoxylin stained material to indicate movement or fixation of the antigen by antisera. By this means he developed a qualitative test for the presence of brucella antibodies in human sera.

Using bovine serum albumin isotopically labelled with I^{131} by the method of Knox and Endicott(2) we have attempted some quantitative measurements of antibody titre to this antigen. Also it has been possible to observe the chromatographic behavior of soluble complexes formed by antigen antibody interactions in the region of antigen excess.

Chromatographic methods. The technic described by Franklin and Quastel(3) for the chromatography of protein mixtures was adapted to the chromatography of varying volumes of I^{131} labelled bovine serum albumin with a standard volume of antiserum using aqueous buffer and salt solutions of suitable pH and ionic strength. The capillary ascent technic of Williams and Kirby(4) using Whatman No. 1 paper strips ¾ inch wide has been used throughout. The antiserum spots usually 10 or 20 mm^3 were placed with calibrated hemoglobin pipettes at marked starting points 2 inches above the lower end of the paper strip. The desired volume of I^{131} labelled antigen solution was superimposed on the antiserum spot by delivery with a Linderstrom-Lang-Holter microburette. The volume usually ranged from 1-20 mm^3. In order to achieve more uniform distribution of the antigen over the antiserum spot a small drop of isotonic saline solution was placed near the tip of the microburette and allowed to wash down the tip of the burette to the paper. There the drop of saline solution radially chromatographed the antigen over the antiserum spot. This was particularly useful when the antigen solution volume was smaller than the antiserum volume. In order to prevent drying of the spots the paper strips were immediately placed in the jar containing the solvent. There they were suspended above the solvent for varying lengths of time before development in order to allow time for the antigen-antibody reaction to proceed. Then the lower ends of the strips were lowered into the solvent and developed for periods varying from 2-6 hours. In the experiments presented 0.15 M sodium chloride was used as the solvent.

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* The technical assistance of Miss Martha C. Havens is gratefully acknowledged.

† The authors are indebted to Dr. William C. Knox for providing the standard precipitin curve on the antiserum tested.

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Measurement of radio-activity. Radiochromatography has undergone rapid evolution in recent years; most methods however have employed segment analysis either by dissection of the chromatogram or radio assay of successive segments along the strip(5). It is believed that an essential improvement of these practices has been achieved by use of a continuous recording instrument.

The measuring device (Fig. 1) consisted of an end window Geiger counter and collimating slit coupled to a counting rate meter and strip chart recording milliameter. In routine procedure, 8 or 10 of the chromatographic strips were taped end to end and rolled onto a feeder spool. After passing the leading end of the strip through the slit guide and taping it to the recorder chart, a continuous record of the radioactivity distributed along the strips could be obtained upon actuation of the chart drive and counter circuits. With the arrangement described, specifications as to chart drive speed and optimal slit width are contingent upon the time constants‡ of the Geiger counter rate meter. These vary in different circuits and sensitivities from values of 1 second to about 1 minute and are minimal for the highest count rate sensitivity range in a given circuit. It is therefore expedient to work wherever possible with a high level of radioactivity in order to minimize the time constant of the rate meter response. When this is done the practical limiting factors are the chart drive speed and slit width.

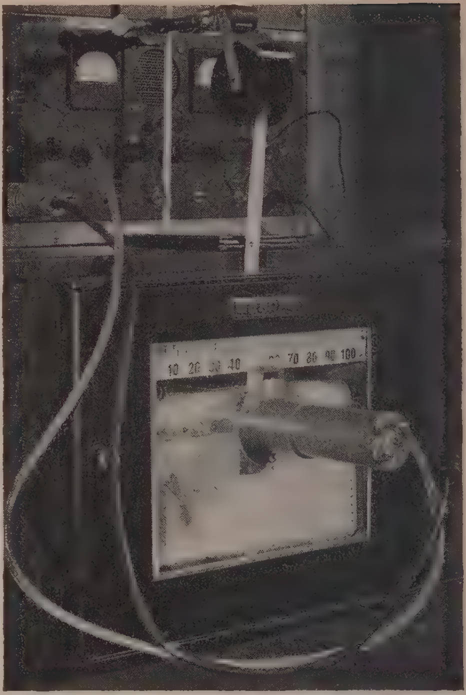

FIG. 1.
Measuring and recording assembly with chromatograms in scanning position.

In order to allow time for the rate meter to respond fully (98%) to a given small segment, $\triangle \chi$, of the strip, it is necessary that this segment remain within the slit space for a period of at least 4 times constants. The limiting slit width, S, is thus definable as the product of the chart drive velocity, V, and a time equal to 4 time constants. That is, $S = V\ (4T)$, where T is the time constant. On the basis of this expression Table I indicates the minimal slit widths compatible with a 98.1% response for various strip velocities at the two sensitivities used in experiments reported here.

The essential validity of these limits was confirmed in experiments with 1 and 10 mm slits at various strip velocities, and the results reported here have been held well within them.

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‡ The "time constant", T, is ordinarily taken to be the time, t, required to reduce the exponent to unity in the expression, $A_t = A_o(1\text{-}e^{-kt})$, whereupon in one such period the response height $A_t = 0.63\ A_o$, or 63% of maximal response.

TABLE I. Slit Widths (mm) Required for 98.1% Full Response for Various Strip Drive Velocities and Response Time Constants.

Strip velocity		Time constants	
(inches/hr)	(mm/sec.)	2.5 sec.	1.0 sec.
30	.21	2.1 (mm)	0.85 (mm)
60	.42	4.2	1.79
90	.64	6.4	2.54
120	.85	8.5	3.39

One additional factor often favors a wider than minimal slit width, namely the statistical advantage of the higher total count thus obtained, and the corresponding reduction in the standard error of measurement. Moreover, as the initial spot of active material placed on the strip is several millimeters in diameter, the ultimate precision of measurement of the gradient along the strip cannot reasonably exceed this order of error; hence a slit width of about half the spot diameter is probably a practical minimum to be sought in this work.

In Fig. 2 are recorded the radioactivity profiles obtained from a series of labelled albumin solution spots placed on a strip of Whatman No. 1 paper with the Linderstrom-Lang-Holter microburette. The areas under the curves were measured with a planimeter and are plotted in Fig. 3 as area in cm^2 against volume delivered to the paper. The volumes ranged from 1.0 to 10.0 mm^3 of a solution containing approximately 3.0 mg/ml of I^{131} labelled bovine serum albumin; the total iodine content was seven atoms of iodine per mole of albumin. Thus the calibration curve represents the range 3.0 to 30.0 μg of protein and appears very nearly a straight line up to 9.0 mm^3. It furnishes a measure of the labelled protein in a circular area with a precision of about 4% for a 1 mm slit and of 2% for a 1 cm slit. It should be pointed out that activity which has been chromatographed along the paper cannot be measured with the same precision as a circular spot except as a linear integral of a constant strip width. Results with a biuret color reaction indicate that the width of the protein spots in the upper part of the strip after development show some variation, so that the geometry of counting is less precise.

Results. In Fig. 4 are shown some radiochromatograms of I^{131} labelled bovine albumin under various conditions. Frame 1 shows that the labelled albumin in 0.15 M sodium chloride solution forms a long heterogeneous streak similar to that found by Franklin and Quastel(3). Similar results were obtained with labelled albumin in a phosphate buffer having a pH of 7.4 and ionic strength of 0.05 to 0.20. However in Frame 2 the activity from 5 mm^3 of labelled albumin solution superimposed on 20 mm^3 of normal rabbit serum is seen moving as a homogeneous single peak and by simultaneous biuret color reaction can be shown to move in the leading portion of the serum protein biuret spot. Little or no activity remains at the starting point of the chromatogram. The R$_f$ value of the activity peak was found to be 0.95.

The different behavior of labelled bovine serum albumin chromatographed alone and

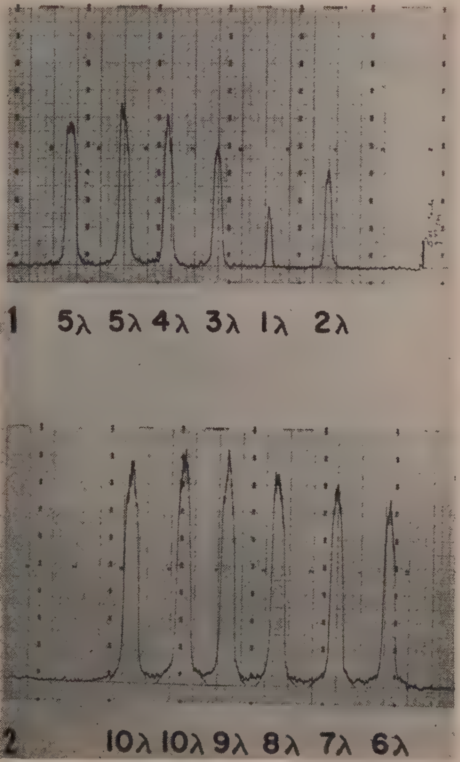

FIG. 2.

Radioactivity profiles of a series of volumes of a labelled bovine albumin solution. The areas under these curves are plotted as a calibration curve in Fig. 3.

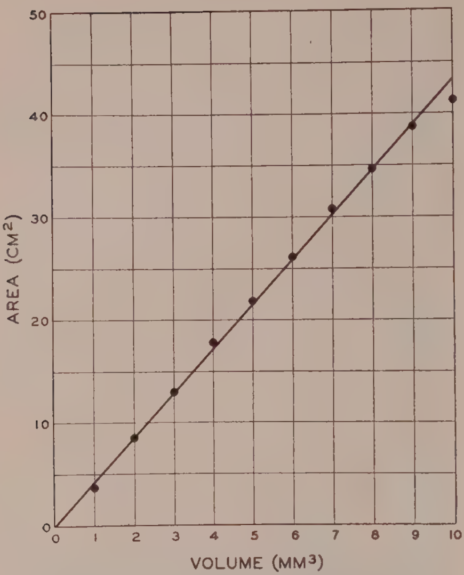

Fig. 3.
Calibration curve obtained from areas shown in Fig. 2.

mixed with normal rabbit serum appears to depend on a critical minimum of total protein placed on the chromatogram. Experiments in which labelled bovine serum albumin was mixed with varying quantities of unlabelled bovine serum albumin have shown the same dependence on a critical quantity of protein. A more complete treatment of these observations will be published in detail at a later date. The serum protein particularly rabbit serum albumin provided by 20 mm^3 of serum is well above the critical quantity necessary for the labelled albumin to move essentially as one spot.

In contrast are the patterns shown in Frames 3-8 which were obtained by superimposing 1 to 6 mm^3 of labelled albumin solution containing approximately 3.0 μg/mm^3 of albumin on 20 mm^3 of rabbit antiserum to bovine serum albumin. It will be seen that in Frames 3-6, representing 1 to 4 mm^3 of labelled antigen, the activity has remained fixed in a single peak at the starting point of the chromatogram. In contrast, when 5 mm^3 of albumin solution were added to 20 mm^3 of antiserum a significant amount of activity moved up the chromatogram. It appeared as (1) a long sloping take-off from the starting point peak (2) a low peak with an R_f value of 0.6 and (3) a small sharp peak moving with an R_f of 0.95 similar to bovine albumin moving on normal rabbit serum. At a level of 6 mm^3, Frame 8 shows a significantly larger total activity which has moved up the paper in peaks with R_f values of 0.28 and 0.95 plus a long valley of activity significantly above the base line. As a preliminary interpretation it seems reasonable to interpret the peaks with R_f values of 0.95 as free albumin or albumin complexed with such small ratios of antibody or other proteins as to have little retarding effect on its movement. The radioactivity peaks retained at the starting points

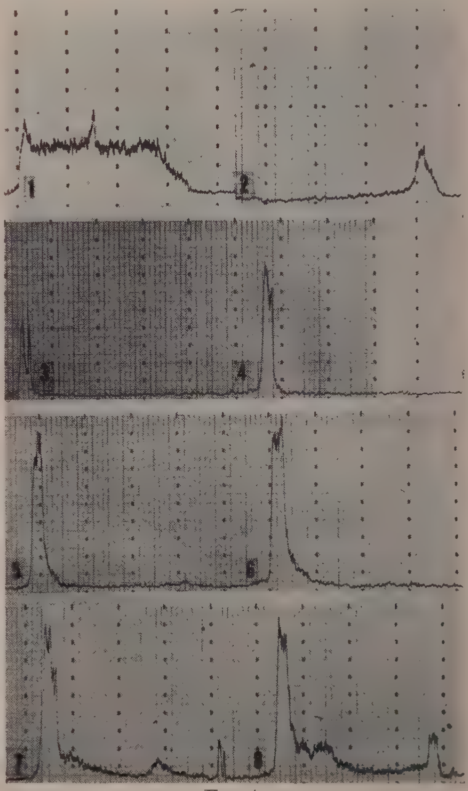

Fig. 4.
(1) Labelled bovine serum albumin in 0.15 M sodium chloride solution. (2) Labelled bovine serum albumin superimposed on normal rabbit serum. (3-8) 1-6 mm^3 of labelled bovine serum albumin solution superimposed on 20 mm^3 of rabbit antiserum to bovine serum albumin. Maximum area was found under curve 6.

of the chromatograms in frames 3-8 in which varying volumes of albúmin solution were added to a constant volume of antiserum appear to represent labelled albumin immobilized on the chromatogram by virtue of its combination with antibody in an insoluble complex. Beyond the equivalence ratio of antigen to antibody, a fraction of the radioactive albumin remains combined in soluble antigen-antibody complexes. These move along the chromatogram, but at rates significantly different from labelled bovine serum albumin on normal rabbit serum.

As a check on the quantitative aspects of the chromatographic titration, the areas under the peaks at the starting points of the chromatograms were measured with a planimeter. There could be measured quantitatively because the spots left at the starting points of the chromatogram were very nearly circular. In order to evaluate the accuracy of the method, aliquots from the same I^{131} labelled albumin solution were used to provide a standard precipitin curve with the same antiserum used in the chromatographic titration. The precipitates were redissolved and made up to volume in 0.25 N sodium hydroxide solution for measurement of ultra-violet absorption at 290 mμ. Since in the chromatographic titration the quantity measured is labelled antigen retained on the starting spot and in the other the total antigen-antibody complex, the absolute values along the curve are not comparable. Therefore the ratios of antigen solution volume to antiserum volume at which maximum retention of antigen and maximum precipitation of antigen antibody complex occurred were compared. The volumes of each were corrected by the appropriate volume and dilution factors to the volume of antigen solution added per ml of antiserum and plotted on the same base line. These data plotted in Fig. 5 show maximum labelled albúmin retention at the starting spot to occur at a ratio of 1.08 ml of albumin solution to 1.0 ml of antiserum as compared to 1.20 ml of the same albumin solution to 1.0 ml of antiserum for the precipitin titration.

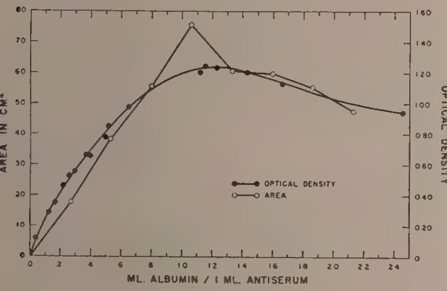

Fig. 5.

Simultaneously plotted are the optical densities at 290 mμ obtained from precipitates from 1 ml of rabbit anti-albumin antiserum and the areas from the starting point peaks from Fig. 4. Both are plotted against the same base line in terms of ml of the same albumin solution against 1 ml of the same antiserum.

Discussion. It has been shown by Eisen and Keston(6) and by Knox and Endicott(2) that lightly iodinated bovine serum albumin shows little deviation from native bovine serum albumin with respect to the precipitin curves obtained with antisera to native serum albumin. However in order to compare the methods as closely as possible aliquots of the same I^{131} labelled albumin solution were used for both titrations. It is encouraging to find that the antigen-antibody ratio at which maximum albumin activity is retained by the antiserum on the starting spot is only 11% lower than the ratio at which maximum precipitation is obtained by a standard method. It should be pointed out that the chromatographic titration provides a measure of the antigen precipitated rather than of the total antigen-antibody complex precipitated as in the usual method of determining a precipitin titre. Hence further check should be obtained using a labelled antiserum prepared in the manner described by Pressman and Sternberger(7). With a bland antigen and a radioactive antiserum a direct measurement of antibody would then be possible. The procedure requires much further study and refinement of method but appears promising not only as a quantitative tool for measurement of antigen antibody reactions, but also in the study of other protein-protein interactions. It may also furnish useful data in

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isolation procedures involving protein mixtures and complexes.

Summary. 1. A continuously recording counter adapted to the scanning of radio chromatograms has been described. 2. This method has been applied to a chromatographic method of demonstrating and measuring the reaction between radioactive iodine labelled bovine serum albumin and its antiserum. 3. Preliminary experiments have shown a reasonable agreement between the zone of maximum antigen fixation obtained by a chromatographic method and the zone of maximum precipitation obtained by the precipitin method. 4. The method appears to have useful possibilities in characterizing soluble antigen-antibody complexes as well as other protein-protein interactions. 5. It has been demonstrated that normal sera have the property of permitting a homogeneous movement of the labelled bovine serum albumin whereas the buffered solutions thus far tried have resulted in streaking of the albumin.

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Effect of 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine and 2,4-diamino-6,7-diphenylpteridine on Chlorguanide-resistant Strain of *Plasmodium gallinaceum.* (18716)

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In previous reports(1,2), resemblances were shown to exist in the biological activities of certain biguanide-type compounds including chlorguanide (Paludrine), certain 2,4-diaminopyrimidines and 2,4-diaminopteridines. The antimalarial activity of these compounds against *Plasmodium gallinaceum* was potentiated by sulfadiazine and partially inhibited by folic acid. It was considered possible that the mode of action of these compounds might be identical, and one would therefore expect that a strain of parasites resistant to one of them would also be resistant to the others. Recently Falco *et al.*(3) have shown that some 2,4-diaminopyrimidines are over 700 times as active as quinine and 60 times as active as chlorguanide aginst *P. gallinaceum.* Such compounds are clearly of potential significance in the treatment of human malaria. Their application in this field might be limited, however, if strains of malaria now resistant to chlorguanide were also resistant to the pyrimidines.

This paper deals with the effect of one representative, 2,4-diamino-5-aryloxypyrimidine and one 2,4-diaminopteridine on a chlorguanide-resistant strain of *P. gallinaceum.*

Methods. Two strains of *P. gallinaceum* were used: a chlorguanide-resistant strain and its "normal" 8A parent strain. According to the criteria used in this laboratory(4) the chlorguanide-resistant strain was, at the time of these experiments, about 16 times more resistant to the activity of the drug than was the parent strain. The hosts were week-old New Hampshire Red chicks weighing 45 to 55 g. The chicks were inoculated at random; each received 16 x 10^6 parasitized erythrocytes intravenously in 0.1 cc of inoculum. Drugs were administered twice daily for 4 days beginning 4 to 5 hours before inoculation. Blood smears were made and stained with Giemsa the morning following the last dose of drug, and parasite counts were made on the basis of parasitized cells per 10^4 erythrocytes. The

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TABLE I. Effect of Chlorguanide, 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine, and 2,4-diamino-6,7-diphenylpteridine on the Parent and Chlorguanide-resistant Strain of *P. gallinaceum.*

	Minimum effective dose, mg/g b.i.d. for 4 days	
Drug	Parent strain*	Chlorguanide-resistant strain*
Chlorguanide	<.002, .002, .002	>.016, .032, .032
Pteridine	<.016, .016	>.064, >.064
Pyrimidine	.002, .004, .002	.002, <.004, .002

* Each result represents one series.

minimal effective dose of a test drug is that amount which will cause a 75% reduction in the mean parasitemia in 5 treated birds as compared with 10 untreated controls. The diaminopyrimidine and the diaminopteridine were tested for antimalarial activity against both strains of the parasite either simultaneously or during the same week.

Experimental. As seen in Table I the minimum effective dose of chlorguanide against the parent strain was about 0.002 mg/g; against the resistant strain approximately 0.032 mg/g. The chlorguanide-resistant strain of the parasite was at least four times as resistant to the activity of 2,4-diamino-6,7-diphenylpteridine as the parent strain. Where 0.016 mg/g was more than the minimum effective dose of the pteridine against the parent strain, 0.064 mg/g was less than the minimum effective dose of the compound against the chlorguanide-resistant strain. The pteridine could not be tested in doses greater than 0.064, since this was the chick's limit of tolerance. On the other hand, 2,4-diamino-5-(p-chlorophenoxy)-6-methyl-pyrimidine was equally effective against both the chlorguanide-resistant and the parent strains of the parasite.

It was unlikely that the mode of action of the pyrimidine and chlorguanide was identical against *P. gallinaceum.* However, it was possible that the two compounds were acting on the same metabolic system of the parasite but at different loci. If this were the case, then one might expect that the two compounds would be synergistic in their antimalarial activity. However, when the two compounds were administered concurrently at less than the minimum effective dose no evidence of synergism was obtained.

Discussion. Since the discovery of chlorguanide (Paludrine) as an active anti-malarial there has been considerable interest in the mode of action of this acyclic biguanide as well as in certain condensed biguanides. The results of this paper and previous findings pertinent to them are summarized in Table II.

The 2,4-diaminopteridines are so similar in their biological characteristics to chlorguanide that the mode of action of these compounds is probably identical. The 2-arylamino-4-alkylaminoalkylaminopyrimidines (the structural precursors of chlorguanide)(11) bear little resemblance to chlorguanide aside from the fact that both compounds have antimalarial activity. The 2,4-diamino-5-aryloxypyrimidines are similar to chlorguanide in certain biological characteristics, but since they exhibit no cross-resistance with chlorguanide, their mode of action is probably different. We have found (unpublished data) that it was impossible to produce resistance to these 5-aryloxy-diaminopyrimidines by methods which easily produced chlorguanide-resistant parasites. The fact that the 2,4-diamino-5-aryloxypyrimidine is effective against chlorguanide-resistant parasites, and that resistance to these compounds is not easily produced may be of considerable importance in the use of these drugs in treatment of human malaria.

It is obvious that the diaminopyrimidines form a heterogeneous group of compounds. Falco *et al.*(3) have pointed out that the antimalarial activity of 2,4-diaminopyrimidines substituted with an alkyl, benzyl or aryloxy

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TABLE II. A Comparison of Biological Activity of Chlorguanide, Certain Pteridines and Diaminopyrimidines.

Activity against		Chlorguanide	Pteridine, 2,4-diamino-6,7-diphenyl	Pyrimidines	
				2,4-diamino-5-aryloxy	2-arylamino-4-alkylamino-alkylamino
P. gallinaceum in vivo	Potentiated by sulfonamides	Yes(6)	Yes(1)	Yes(2)	No(2)
	Inhibited by folic acid	” (7)	” (1)	” (2)	—
	Cross-resistant to chlorguanide	—	” (5)	No(5)	No(10)
Lactobacillus *in vitro*	Potentiated by sulfonamides	—	” (9)	—	—
	Inhibited by folic acid	Yes(8)	” (9)	Yes(8)	—

group in the 5-position is decreased by substitution on the 2- and/or 4-position amines. The antimalarial activity of the diaminopyrimidines with no or an alkyl substitution on the 5-position is dependent on substitutions on the 2- and 4-position amines. These findings accentuate the differences noted in Table II.

Summary. A chlorguanide-resistant strain of *Plasmodium gallinaceum* was cross-resistant to 2,4-diamino-6,7-diphenylpteridine. The same chlorguanide-resistant strain was not cross-resistant to 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine. Chlorguanide and 2,4-diamino-5-(p-chlorophenoxy)-6-methylpyrimidine were not synergistic in their antimalarial activity.

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Chemotherapeutic Effects of a Naphthoquinonimine on Infection of Mice with Columbia-SK Group of Viruses.* (18717)

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Schnitzer, Buck, and Steiger(1) described the chemotherapeutic effects obtained with a certain naphthoquinone derivative (compound Ro 2-3532, *i.e.* 3-(1,4-dihydro-3-hydroxy-4-oxonaphthylideneamino) N - [β-(β-hydroxyethylamino)-ethyl] benzamide) in mice infected with Col-SK or MM virus. This substance is not soluble in water but may be rendered highly water-soluble through coupling with a reducing agent such as sodium bisulfite. This process is reversible by oxidation. The solubilized compound is designated as Ro 2-3532/1. Through cooperation with the above workers we were able to examine the chemotherapeutic properties of this substance in this Laboratory. The results are presented here. In chemotherapeutic studies of a neurotropic viral disease it is obviously of advantage to limit the experimental approach to efforts designed to restrain the virus from entering the central nervous system rather than to attempt to influence is intraneural spread. Hence, the Columbia-SK type of virus was chosen for this work. As is well-known, Col-SK virus—in contrast to Yale-SK, Lansing, or MEF virus—possesses a high and constant degree of infectivity in rodents by various peripheral channels of inoculation.

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* Aided by a grant from the Sister Elizabeth Kenny Foundation.

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Equally important is that this virus is also excreted through the alimentary tract in orally infected mice.

Materials and methods. In most of the experiments Col-SK virus was used as the test strain. On several occasions, however, the drug was also examined for its ability to prevent the infection of mice with two antigenically related viruses, *i.e.* EMC virus and F virus. The latter virus was recently isolated by Koch(2) as a primary rodent-pathogenic agent from the spinal fluid of a fatal case of undiagnosed human illness, characterized by vague central nervous system symptoms and definite interstitial myocarditis. The virus was subsequently identified by Bieling(3), through immunological methods, as belonging to the Col-SK group of viruses. We are indebted to Dr. R. Bieling, Marburg, for kindly sending us a sample of the F strain.† The choice of all 3 viruses was motivated by the fact that these agents, despite their common serological and biological properties, possess in rodents different endpoints of potency readily demonstrable in carefully conducted titration experiments. It was realized that in such chemotherapeutic experiments the reliability of the results may be decisively affected by possible variations in the purity of the drug as well as by possible fluctuations in the virulence of the virus. Chemical uniformity could be assured by using only pre-tested lots of the drug and making up the desired solutions of the soluble compound in distilled water, immediately prior to injection, so as to prevent oxidative deterioration. It is more difficult to satisfy the second consideration adequately. We decided to use in our tests only viral brains which had been harvested on the day of the experiment from carefully selected paralyzed passage mice infected intracerebrally 48 hours before. While pools of concentrated viral brain suspensions frozen and stored in dry ice, are acceptable as seed within certain limits (Schnitzer *et al.*)(1) previous experience has convinced us that such preparations not only contain appreciably less extractable infectious material, but that further deterioration of infectivity may occur irregularly and unpredictably over a period of time. That technical differences in the preparation of viral suspensions play an important part in determining their ultimate potency is also suggested by reference to the wide range in LD_{50} endpoints recorded by various workers who titrated Col-SK or MM virus intraperitoneally in mice. This range extends from $<10^{-8}$(4) or $<10^{-7.9}$(5) to values lying between 10^{-5} and $10^{-6.6}$(6-8) or between 10^{-6} and 10^{-7}(9).

Experimental work with the drug proceeded along the following basic lines of investigation: (1) evaluation of the protective effects obtainable when a single standard dose of drug (5 mg in 0.5 ml volume) is given intraperitoneally a few minutes following infection with 0.1 ml of various virus dilutions by the same route; (2) evaluation of the protective effects obtainable when a single dose of drug is given subcutaneously (5 mg) or intravenously (1 mg) a few minutes following intraperitoneal infection with 0.1 ml of various virus dilutions. Swiss albino mice weighing 9-12 g were used. To ensure an even distribution of the virus in the experimental animals, large numbers of mice were first infected at random with each successive virus dilution and then divided into equal smaller groups for subsequent treatment or for controls. The results obtained with the 3 viruses appear in

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† In preliminary experiments in this laboratory the F virus has proved to be definitely pathogenic for rhesus monkeys in serial passage, producing flaccid paralysis and typical poliomyelitic lesions in the spinal cord.

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Table I. Quantitative evaluation of the data is given in Fig. 1.

TABLE I. Effect of a Single Dose of Compound Ro 2-3532/1 Administered at Time of Intraperitoneal Infection of Mice with Col-SK, F, or EMC Virus.

	Route of inj.			Virus dilutions							
Virus	Drug	Virus	Exp.	10^{-5} (%)	10^{-6} (%)	10^{-7} (%)	10^{-8} (%)	10^{-9} (%)	10^{-10} (%)	10^{-11} (%)	10^{-12} (%)
Col-SK	i.p.	i.p.	I-VII	16/16(100)	41/76 (54)	19/72 (26)	5/56 (9)	1/87 (1)	0/19 (0)		
	s.c. or i.v.	,,		20/20(100)	19/20 (95)	15/20 (75)	8/30 (27)	0/20 (0)	0/19 (0)		
	Controls	,,	Totals	30/30(100)	70/70(100)	68/70 (97)	62/69 (89)	37/60(62)	13/50(26)	1/21 (5)	
F	i.p.	,,	I-IV	28/29 (96)	22/30 (73)	5/30 (17)	2/29 (7)	0/29 (0)	0/29 (0)	0/19 (0)	
	s.c.	,,		10/10(100)	8/10 (80)	12/20 (60)	5/20 (25)	2/18(11)	0/20 (0)		
	Controls	,,	Totals	30/30(100)	30/30(100)	40/40(100)	40/40(100)	29/40(72)	18/40(45)	7/40(17)	0/10(0)
EMC	i.p.	,,	I-II	19/20 (95)	13/20 (65)	8/20 (40)	0/10 (0)	0/10 (0)			
	s.c.	,,		20/20(100)	15/20 (75)	11/20 (55)	2/10 (20)	0/10 (0)			
	Controls	,,	Totals	20/20(100)	16/20 (80)	13/20 (65)	5/20 (25)	1/9(11)			

Numerator = No. of mice paralyzed; denominator = No. of mice infected.
Dose of drug = 0.5 ml of 1% sol. (5 mg) i.p. or s.c.; 0.5 ml 0.2% sol. (1 mg) i.v.; dose of virus = 0.1 ml virus dilutions i.p.

It will be seen from Table I that a single dose of 5 mg of the drug administered intraperitoneally, was sufficient to protect a high percentage of the mice against infection with multiple paralytic doses of Col-SK virus injected simultaneously by the same route. Experiments in which the virus was injected intraperitoneally, but the drug was injected subcutaneously or intravenously, showed likewise definite protection, though inferior to that obtainable when both were given by the same route. This protection could be demonstrated to a similar degree in repeated experiments. No protection whatsoever was observed in mice which had been infected by the intracerebral or intramuscular route even when minimal doses of virus and maximal doses of drug were injected simultaneously at the same portal of entry. Experiments with orally infected mice fed a diet containing the drug in 2% concentration gave inconclusive results in repeated tests. Work is in progress to determine whether the drug has any effect on the fecal excretion of the virus. A clear-cut protection against multiple paralytic doses of virus was also demonstrable when the drug was used in mice infected intraperitoneally with the F virus. Again, these effects could be elicited when drug and virus were injected by the same or by separate peripheral routes. In the case of EMC virus, the effect of the drug under corresponding experimental conditions was much less marked, even when drug and virus were given by the same route. When a precise comparison of the protective action of the drug against the 3 viruses is made and the data are analyzed mathematically (Fig. 1), it will be found that the drug protected evenly against at least 1000 LD_{25}, LD_{50} or LD_{75} of Col-SK and F virus, whereas the protection observed against EMC virus extended only from 5 to about 10 LD_{25}, LD_{50} or LD_{75}.

Attempts were made in a few experiments to determine the effective quantitative range of the drug against infection with variable doses of Col-SK virus and also to investigate the time limits following infection at which drug treatment still prevented paralysis and

TABLE II. Effect of Intraperitoneal Injection of Compound Ro 2-3532/1 on Intraperitoneal Infection of Mice with Col-SK Virus.

Virus Dilutions	Relation between dose of drug and protection Dose of drug given at time of infection 2.5 mg	1.25 mg	0.5 mg	0.25 mg	Relation between interval of time and protection Interval between infection and inj. of 5 mg of drug 15 min.	30 min.	60 min.	120 min.	Controls
10^{-7}	3/20	1/10	8/20	10/20	11/19	10/18	11/20	17/20	30/30
10^{-8}	0/20	2/10	6/19	7/20	1/17	0/17	1/19	4/20	25/29
10^{-9}	0/18	0/10	1/19	4/19	0/15	0/19	0/13	4/20	14/30

death. In all these tests drug and virus were given by the same route, *i.e.* intraperitoneally. The results are shown in Table II. It appears from Table II that significant protection was still evident with quantities of drug ranging from one-half (2.5 mg) to one-tenth (0.5 mg) of the standard dose, provided the amount of virus did not exceed 10 LD_{50}. As far as the time limits of drug efficacy are concerned, the results suggest that the standard dose of drug (5 mg) still exerted a significant degree of protection up to and including 1 hour following intraperitoneal infection with 10 LD_{50} doses of virus. The above data are based on a comparatively small number of animals; therefore, any conclusions on these important points must be tentative at present.

It seemed next of interest to determine whether or not drug-protected animals had acquired any immunity towards reinfection with Col-SK or with F virus. In these tests a total of more than 300 surviving drug-treated mice were challenged, 2 weeks after their initial injection, with a moderate dose (10^{-5} *i.p.*) of the homologous virus. Suffice it to say, that all these animals, without exception, developed paralysis like the corresponding controls. Thus, there was no question that drug treatment had failed to leave the protected animals with any appreciable immunity. The following experiments deal with efforts to analyze the probable mode of action of the drug. From the outset, it seemed unlikely that the drug should be capable of modifying the contact between virus and susceptible cells. Experiments in which the

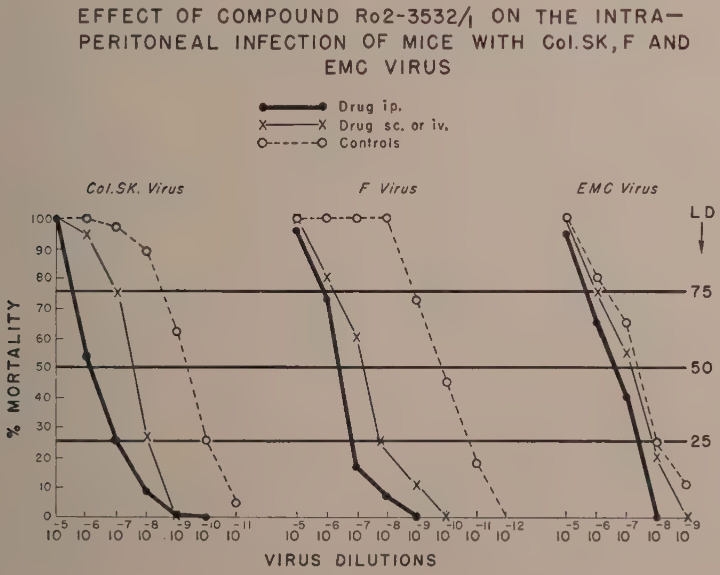

FIG. 1.

drug was used to study a possible hemagglutination-inhibitory action on sheep red cells failed to provide a clear answer, however, because the drug exercised a marked destructive effect upon the erythrocytes. Experiments were then designed to investigate the fate of the virus in the drug-treated animals. Groups of mice which had received small doses of virus (10^{-6} or 10^{-7}) and the standard dose of drug (5 mg), both by intraperitoneal injection, were killed at intervals ranging from 2 hours to 24 hours. The peritoneal cavity was washed out with 1 ml of saline and the washings were transferred to new mice by intramuscular injection. At the same time 0.25 ml of blood was collected from the killed animals and injected intramuscularly into new mice. Control mice, which had received virus but no drug, were killed at the same intervals and their peritoneal washings and blood were transferred to new mice in the same way. The results of several tests may be summarized by saying that in the case of untreated control animals live virus could be recovered regularly at all intervals from the peritoneal cavity and at the later stages also from the blood, whereas in the case of the drug-treated mice virus was only sporadically found in the peritoneal cavity at the early intervals and the virus either failed to appear later in the blood stream or the viremia was definitely delayed. Our data are in essential agreement with the basic information collected by Evans and Chambers(10) on the mechanism of infection with MM virus following peripheral infection of hamsters. They also offer a clue as to why the drug-treated surviving animals had remained fully susceptible to reinfection.

The sum total of these experiments seemed to suggest that the drug exercised a prompt and profound antiviral action *in vivo* as the result of direct contact with the virus, but the data, by themselves, are hardly sufficient to prove the point. Unfortunately, the problem cannot be approached to complete satisfaction by studying the action of the drug on the virus in *in vitro* prepared mixtures because it is difficult or impossible to separate virus from the drug by physical methods such as dialysis, sedimentation or filtration. However, a simple method which seemed adequate enough to yield reliable information was utilized in some experiments. It consisted in setting up mixtures of 0.5 ml virus dilutions (10^{-2} or 10^{-4}) in combination with 0.5 ml drug solutions (5% or 0.5%), letting these mixtures stand in the icebox for various lengths of time (0 minutes, 30 minutes, 60 minutes) and then making ten-fold serial dilutions of these mixtures which were carried into final concentrations of the drug beyond its known effective range but which were still within the range of expected viral infectivity. These mixtures were then injected, in 0.1 ml volume, into mice by the intramuscular route to test for the presence of viable virus. Control mixtures were set up with distilled water and handled the same way. The results showed that a virus dilution of 10^{-2} was not influenced by a 5% drug concentration, except after 60 minutes contact, when some mice survived with 10 LD_{50} doses of virus (10^{-7}); however, a virus dilution of 10^{-4} in combination with a 0.5% drug concentration apparently had suffered a definite loss of infectivity at the 30 and 60 minute intervals since mice survived with 100 LD_{50} doses of virus (10^{-6}, 10^{-7}). Curiously enough, the amount of virus which could be suppressed *in vitro* was of the same order as that which responded to *in vivo* administration of the drug. These results would indicate that comparatively small doses of the drug are capable of inactivating, in a more or less fixed ratio, moderate though not large amounts of virus upon direct contact *in vitro*.

Discussion. It appears from our data that we were able fully to confirm the observations reported by Schnitzer *et al.*(1), namely, that reproducible chemotherapeutic effects may be obtained in mice with compound Ro 2-3532/1 following intraperitoneal infection with multiple paralytic doses of Col-SK virus. These protective effects, however, being limited to the extraneural phase and early intervals of the infection are probably more chemoprophylactic than chemotherapeutic in the true sense of the word. Our experiments show, in addition, that the drug exerts a similar definite

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10. Evans, C. A., and Chambers, V. C., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 436.

protective action on the corresponding infection with F virus. On the other hand, the results obtained against infection with EMC virus are so slight that one must question the significance of such minimal protection. The magnitude of the chemotherapeutic effects against Col-SK and F virus seems to be quite constant, depending critically upon the quantitative relationships between virus and drug and approximating occasionally the "all or nothing" type of a chemical reaction. This remarkable regularity in the chemotherapeutic system makes it possible to predict, with fair accuracy, the outcome of an experiment. Expressed in LD_{50} of Col-SK virus, which the drug-treated animals tolerated without developing paralysis, the protection obtained in our experiments was significantly in excess of that recorded by the original investigators. Obviously, this difference was caused by the use of more virulent virus preparations in our hands. It is difficult, however, to explain satisfactorily the fixed maximum level of virus which the drug brought under control, irrespective of the prevailing endpoints of infectivity. One possible assumption would be that during storage of frozen virus a certain proportion of active virus particles is converted into inactive virus particles, both functioning as physical or chemical substrate in association with the drug. A similar observation relating to discordance between viral mass and viral infectivity—albeit within a much lower order of concentration—was recently reported for the Lansing virus(11). The absence of any convincing action of the drug on EMC virus, which is in marked contrast with its pronounced action on Col-SK and F virus, requires some comment. Since EMC and Col-SK virus are antigenically very closely related one may have to look for physical rather than chemical differences between the two viruses for an explanation. Measurements of size for the three viruses in this group, *i.e.* Col-SK, MM and EMC, have been estimated as follows by various authors: Col-SK virus 9-14 mμ (by ultrafiltration), 20-35 mμ (by electronmicroscopy), 130 S (sedimentation constant by ultracentrifugation); MM virus 11-16 mμ (by ultrafiltration), 10-20 mμ (by electronmicroscopy), 125 S (sedimentation constant by ultracentrifugation); EMC virus 35 mμ (by electronmicroscopy), 153 S (sedimentation constant by ultracentrifugation). No corresponding figures are available for the F virus. The data suggest that EMC virus is slightly larger than Col-SK or MM virus, a fact which may be of some significance in connection with a possible adsorption of the drug on the virus particle.

When one contrasts the chemotherapeutic effects produced by compound Ro 2-3532/1 with the protective effects obtainable through the use of Burnet's RDE (receptor destroying enzyme) preparations from *Vibrio cholerae* cultures(12) against the same infection(13, 14), it becomes evident that one is confronted with very different mechanisms of operation. In the case of the drug, all available evidence points to the fact that such protective action as is demonstrable in these tests lasts only as long as the virus has not begun to multiply on susceptible cells. In other words, since there is nothing to suggest that the drug will effectively compete with virus attachment to cells, or retard intracellular growth, one is led to the assumption that infection is aborted by some direct action of the compound on "resting" virus which is in process of transit from a suitable peripheral portal of entry to its ultimate locus of multiplication. In keeping with this assumption is the rapid sterilization of the infected primary site of infection by the drug and the absence of any demonstrable immunity in the drug-treated surviving animals. In other words, one is dealing essentially with a disinfectant, but a disinfectant which has the unique ability of rendering virus non-infectious *in vivo* on an apparently quantitative basis, without causing injury to tissues and without impairment of its antiviral action by body fluids. In the case of RDE treat-

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11. Bennett, B. L., and Salk, J. E., *J. Immunol.*, 1951, v66, 277.

12. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Br. J. Exp. Path.*, 1946, v27, 228.

13. Verlinde, J. D., and De Baan, P., *Ann. Inst. Past.*, 1949, v77, 632.

14. Jungeblut, C. W., *Bull. N. Y. Acad. Med.*, 1950, v26, 571.

ment, the inhibition of hemagglutination *in vitro* suggests a blocking action on cell receptors rather than any form of direct interaction between Col-SK virus and RDE principle. This concept is further supported by the initial presence of live virus at the portal of entry of the infected animal which has received RDE by the same route, as well as by the fact that a substantial degree of immunity to reinfection can be demonstrated in a majority of the surviving animals. It seems plausible that further experiments in which the cell-protective action of a potent enzymatic principle, such as RDE, is combined with the direct antiviral action of a colloidal dye of the Ro 2-3532 type, should permit the realization of even more powerful chemotherapeutic effects over a longer range of the infectious process.

Summary and Conclusions: The chemotherapeutic effect of a certain napthoquinonimine (compound Ro 2-3532/1) was studied on the peripheral infection of mice with Col-SK virus, F virus and EMC virus. It was found that a well tolerated single dose of 5 mg of the drug, administered intraperitoneally, was sufficient to protect a high percentage of the mice against infection with multiple paralytic doses of Col-SK or F virus injected simultaneously by the same route. Experiments in which the virus was injected intraperitoneally but the drug subcutaneously showed likewise a definite degree of protection though inferior to that observed when both were given by the same route. Significant protection could also be obtained up to and including 1 hour after intraperitoneal infection with small doses of virus. Experiments in which the drug was tested for its chemotherapeutic action against EMC virus showed only a minimum degree of protection considered to be insignificant. Some attempts were made to analyze the probable mode of action of the drug on Col-SK virus. It is concluded that protection occurs not because of cell-receptor blockade but that the virus is rendered non-infectious by some form of direct interaction between virus and drug.

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Chemotherapeutic Effect of 2-hydroxy-1,4-naphthoquinonimine on Infections of Mice with Col SK Virus. (18718)

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These studies were prompted by the observations of Woods and Rusoff(1) and Bieter and Wright(2) according to which the tetrazo dyestuff trypan red exerted a marked prophylactic effect on the intra-abdominal infection of mice with MM virus. These findings could not be reproduced in our experiments thus confirming the work of Hammon, Aird and Sather(3). Nevertheless, experimental work with various azo dyestuffs was included in the screening program with Col SK and MM virus which has been carried out in this laboratory. These azo dyestuffs, most of them derived from sulfonamides, also failed to show any protective effect on the infection with the above viruses. It was, however, observed that compounds of a series of 2-hydroxy-1,4-naphthoquinonimines possessed more or less marked activity, if administered to mice previously infected with MM- or Col SK-virus. A group of 160 compounds of this type has been tested during recent years and 67 members of the series were found to be active in the viral infection. In the present note we shall limit ourselves to the description of the chemotherapeutic property of one representa-

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1. Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, v82, 297.
2. Bieter, R. N., and Wright, H. N., *Poliomyelitis* 1949, p. 276. (J. B. Lippincott Co., Philadelphia)
3. Hammon, W. McD., Aird, R. B., and Sather, G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 511.

tive compound which illustrates particularly well the general type of activity encountered in this group of compounds.

The compound is 3-(1,4-dihydro-3-hydroxy-4-oxonaphthylideneamino)-N-[β-(β-hydroxyethylamino)-ethyl]benzamide (Ro 2-3532), a substance of orange red color (U. S. Pat. 2,551,647). The compound was tested as the water soluble bisulfite derivative (Ro 2-3532/1) primarily because it was more convenient in experiments with small laboratory animals to use solutions rather than suspensions of the insoluble product (Ro 2-3532). The latter, however, was found to possess a similar type of basic activity.

Materials and methods. All experiments were carried out in young (16-18 days) mice of 10-12 g weight drawn from one colony. Animals from 2 other breeders were occasionally tested and found to respond equally well to the infection and the therapeutic treatment.

Strain of Col SK-virus. The strain of Col SK-virus was kindly given to us by Dr. C. W. Jungeblut, Department of Bacteriology, College of Physicians and Surgeons, Columbia University. The strain was passed through mice by intracerebral infection and for these experiments pools of infected brains were prepared and kept in the frozen state. Each viral pool consisted of the brains of about 60 mice infected intracerebrally 2 days before. Mice with the symptoms of flaccid paralysis were sacrificed, the brains harvested, weighed and emulsified with saline in a Waring blendor to give a 1-5 suspension. The suspension was then dispensed in 1.0 ml amounts into sterile rubber capped vials, quickly frozen in a dry ice-acetone mixture and stored at –10°C. For the experiments the contents of one ampul were thawed, and the desired virus dilutions prepared with saline. Since at least 2 and frequently 3 experiments were carried out every week, the pools were generally not used for longer than 8 weeks. The virulence of the different pools of infective brain was consistent and did not change appreciably during the comparatively short time of storage. An inoculum of 0.1 ml 10^{-6} or $10^{-5.7}$ was used in the majority of the experiments. These amounts were fatal for 90-100% of the infected animals and represent 10 to 20 50% lethal doses. The intra-abdominal infection of mice with 0.1 ml 10^{-7} dilution resulted in about 50% fatalities (*e.g.* 68 mice out of 125 = 54.5%) while 0.1 ml of a 10^{-8} dilution of brain suspension was fatal for only 12% of the animals.

Compound Ro 2-3532/1. The compound was first tested as a stabilized solution, but later on freshly prepared solutions were used. The substance dissolves readily with orange red color in distilled water; since the solutions are not stable, they were prepared shortly before use and not kept for longer than one to two hours. Dilutions in distilled water were prepared to contain the desired amount of drug in 0.5 ml.

Experimental procedure. In the standard experiments, groups of 10-20 mice were infected intra-abdominally with 0.1 ml of 10^{-6} or $10^{-5.7}$ diluted brain suspension, corresponding to 10-20 LD_{50}. During the time of the inoculation the tubes containing the dilutions of brain suspensions were kept in ice water. Immediately following the infection, the mice received a *single* intra-abdominal or subcutaneous injection of the drug in 0.5 ml per 10 g mouse. Single administration of the compound was used since numerous experiments with members of this class of compounds had demonstrated that repeated treatment with these substances, either on the same day or on consecutive days, did not result in more marked activity. Groups of untreated mice remained as controls after experience had shown that the injection of saline had no influence on the course of the infection. Titration of the virulence of the virus by including groups of mice with lower infective doses was carried out from time to time but was not routinely added to all experiments. Treated and untreated mice were observed to their death or, in case of survival, for a 21-day period, and symptoms of the infection were daily recorded. As a rule the first symptoms appeared 3 to 4 days after the infection and death occurred within the first 10 days. After that time no essential changes were observed in the experiments. Modifications of the site of the infection, as well as variations in the dose of the drug and its route of administra-

TABLE I. Effect of a Single Intra-abdominal Administration of Ro 2-3532/1 Immediately after the Intra-abdominal Infection of Mice with Col SK Virus.

		Drug		Treated mice			Control mice			
Viral	Virus	dose	No. of		survivors			survivors		P
pool No.[*]	LD_{50}	mg/kg	tests	No.	No.	%	No.	No.	%	=
6	10-20[†]	250	6	59	32	54.2	60	4	6.7	<.001
7	”	250	7	69	52	75.5	70	2	2.8	<.001
3	”	500	17	209	179	85.6	220	15	6.8	<.001
4	”	500	23	387	308	79.6	418	5	1.2	<.001
5	”	500	24	300	265	88.3	320	18	5.6	<.001
7	”	500	17	222	160	72.0	205	12	5.8	<.001
5	100[‡]	500	2	20	2	10.0	20	0	0.0	ns[§]
6	”	500	3	30	11	36.7	30	1	3.3	<.01
7	”	500	3	40	1	2.5	30	0	0.0	ns

[*] Pools 3, 4, 5, 6, 7 were prepared on 6-1, 8-4, 9-22, 11-9 and 12-13-50 respectively and used to 8-8, 9-22, 10-30, 12-18-50 and 2-22-51 respectively.

[†] Dilution 10^{-6} or $10^{-5.7}$ of the virus suspension.

[‡] Dilution 10^{-5} of the virus suspension.

[§] ns = not significant.

TABLE II. Synopsis of the Data on Table I.

			Treated mice			Control mice			
Virus	Drug dose	No. of		survivors			survivors		P
LD_{50}	mg/kg	tests	No.	No.	%	No.	No.	%	=
10-20	250	13	128	84	65.6	130	6	4.6	<.001
10-20	500	81	1118	912	81.6	1163	50	4.4	<.001
100	500	8	90	14	15.6	80	1	1.3	<.01

tion will be mentioned in the experimental part.

Experimental. Toxicity. The toxic effect of a single injection of the compound Ro 2-3532/1 was determined in mice and rabbits. The LD_{50} for adult white mice of 16-20 g was 1070 mg/kg for the subcutaneous route, 818 mg/kg for intra-abdominal, and 422 mg/kg for intravenous injection. The young mice as used in the viral experiments were not essentially more sensitive; the intra-abdominal or subcutaneous injection of 500 mg/kg which was well tolerated by the older animals was also harmless for the younger mice. The LD_{50} for albino rabbits of 1.8-2.0 kg was found to be 168 mg/kg by the intravenous route. The toxicity of Ro 2-3532/1 might have been influenced by the presence of an excess of bisulfite in the comparatively high doses used. That sodium bisulfite can exert marked toxic effects has recently been shown by Hoppe and Goble(4). The insoluble naphthoquinonimine given as a suspension in gum arabic was less toxic. Amounts larger than 5.0 g/kg were tolerated *per os*, while the LD_{50} of an intra-abdominal injection was 1674 mg/kg, *i.e.* more than double the value found with the soluble derivative.

Experiments with Col SK-virus. In Table I a survey of the results of all experiments with the various viral pools is given and the totals are summarized in Table II. It is evident from these figures that with an infective dose of 10-20 LD_{50} which was fatal for an average of 95% of the untreated controls a significantly high survival rate was observed, particularly with the dose of 500 mg/kg. The variation of the percentage of survivors in the individual experiments was in no instance higher than 20%, indicating that the range of activity in the different experiments varied from an occasional minimum of 50% to a maximum of 90-100%. The lower treatment dose of 250 mg/kg was occasionally (*e.g.* with pool 6) of lower activity. Mice infected with higher virus doses, *e.g.* with 10^{-5} diluted brain suspension corresponding to 100 LD_{50}, did not respond satisfactorily to the treatment with the compound, although in one group of experiments a significant survival rate was found.

The doses of Ro 2-3532/1 used in these

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4. Hoppe, J. O., and Goble, F. C., *J. Pharmacol.*, 1951, v101, 101.

TABLE III. Evaluation of the PD_{50}† of Ro 2-3532/1 in the Intra-abdominal Infection with Col SK-virus (10 LD_{50}).
Infection: 0.1 ml 10^{-6} brain suspension. Treatment: single intra-abdominal injection.

Dose, mg/kg	Survival ratio*	% survivors	PD_{50}, mg/kg†
10.0-12.5	11/40	27.5	
25.0	26/70	37.1	
50.0	39/70	55.7	44.4
100.0	44/70	62.9	
250.0	53/59	89.8	
500.0	30/30	100.0	
Controls	1/50	2.0	

* No. surviving/No. infected mice.

† 50% protective dose, calculated according to Reed and Muench.

experiments were multiples of the minimal active dose which was evaluated in a group of 4 experiments with almost identical results. The data were, therefore, combined in Table III. This table shows that as little as one-tenth and one twentieth of the well tolerated dose of 500 mg/kg exerted a marked effect if given immediately after the infection and at the same site. Significant survival rates ($p = < 0.001$) were still observed with a dose of 25 mg/kg. Smaller doses of 10-12.5 mg/kg were no longer significantly active.

Two questions arose from the observations described so-far: Is the effect of Ro 2-3532/1 influenced by (1) the time of treatment and (2) by the site of the drug administration? From the experiment given in Table IV it is seen that a considerable drop of activity occurred, if a single dose of Ro 2-3532/1, 500 mg/kg was administered after a 15 to 60 minute interval between infection and treatment. After 90 minutes and later no convincing effect was seen at all. Although it is evident, that the course of the infection was noticeably changed in the treated animals and the death of the unsuccessfully treated animals was delayed, the survival rate was, if at all, only on the borderline of significance. Attempts to protect mice by giving Ro 2-3532/1 at different intervals before the infection have failed. The dose of 500 mg/kg which fully protected the animals if given simultaneously failed to show any effect even one hour before the infection.

On the other hand, it was possible to control an intra-abdominal infection by treatment with Ro 2-3532/1 remote from the site of the infection. Table V contains the results of a series of experiments in which a single large dose (500 mg/kg) of the compound was given subcutaneously following the intra-abdominal infection with 10-20 LD_{50} of the virus. Significant survival rates of an average of about 50% were observed in all groups of experiments. It was interesting to note that this property of Ro 2-3532/1 was not very common in the class of naphthoquinonimines. Even chemically closely related substances which exerted approximately the same activity as Ro 2-3532/1 when given intra-abdominally, *i.e.* at the site of the viral infection, failed to show an effect by the subcutaneous route. No explanation for the exceptional finding with Ro 2-3532/1 can be offered at the present time.

Practically the same results were obtained in experiments with MM virus: In 8 tests using an intra-abdominal infection of 10 LD_{50} 73 out of 79 mice survived after intra-abdominal treatment with 500 mg/kg (= 92%), 40 out of 80 mice survived after the subcutaneous administration of the same dose (= 50%) while 10 out of 122 untreated control animals remained alive (8.4%). Of other viruses only influenza virus type A (PR8-strain) was tested. Repeated subcutaneous treatment with Ro 2-3532/1 was without effect in the intra-nasal infection of mice.

According to our present experience the effect of Ro 2-3532/1 and the related compounds is limited to the intra-abdominal infection of mice with Col SK or MM virus. All attempts to influence intracerebral, intramuscular, subcutaneous and plantar infections with these viruses have failed so far. This seems to indicate that when virus is introduced in the peritoneal cavity, conditions are favorable to permit a sufficient exposure of the virus to active concentrations of the compound thus preventing infection of the central nervous systems in the majority of animals.

Very little information is at present available as to the possible mechanisms of activity of the naphthoquinonimines in the Col SK or MM infection. There is no indisputable evidence that Ro 2-3532/1 and related com-

TABLE IV. Effect of a Single Intra-abdominal Injection of Ro 2-3532/1 (500 mg/kg) at Different Intervals after the Intra-abdominal Infection with 10 LD_{50} of Col SK-virus.

Interval,* min.	No. of mice	No. of survivors after ... days 3	4	5	6	7	8	9	% survivors†	P =
0	30	27	26	26	26	26	26	26	90	$<.001$
15	30	28	17	13	13	13	12	11	36	$<.05$ $>.02$
30	30	29	26	21	19	18	15	15	50	$<.01$
60	30	26	22	17	14	12	12	12	40	$<.02$ $>.01$
90-120	40	36	28	12	9	9	9	9	23	n.s.‡
180-240	20	15	5	2	1	0			0	
Controls	30	27	16	6	5	4	3	3	10	

* Between infection and treatment.
† After 21 days.
‡ n.s. = not significant.

TABLE V. Effect of a Single Subcutaneous Administration of Ro 2-3532/1 on the Intra-abdominal Infection of Mice with Col SK-virus.

Infection: 10-20 LD_{50}*. Treatment: 500 mg/kg, immediately after infection.

Pool No.	No. of tests	Treated mice No.	Treated mice survivors No.	%	Control mice No.	Control mice survivors No.	%	P =
3	1	20	12	60.0	20	2	10.0	$<.01$
5	5	60	37	61.7	60	2	3.3	$<.001$
7	6	90	40	44.5	70	2	2.9	$<.001$
Totals	12	170	89	52.3	150	6	4.0	$<.001$

* 10^{-6} dilution.

pounds exert an effect on the virus *in vitro.* Experiments with direct exposure of the virus to Ro 2-3532/1 *in vitro* were carried out by suspending infective brain in a final dilution of 10^{-2} in 0.5% to 2.5% solutions of the substance. These mixtures were kept at room temperature for 2 hours. The inoculum was then freed of the drug by washing and dilutions of 10^{-3}, 10^{-4}, 10^{-5} were prepared and injected intra-abdominally into groups of 5 mice for every dilution. A viral brain suspension in saline was treated in an identical manner and mice were infected with this material. In 2 out of 5 experiments all mice infected with the 10^{-5} dilution of the virus exposed to Ro 2-3532/1 survived. This occurred without relation to the drug concentration, *i.e.* in one instance after exposure to the 2.5%, in the other after exposure to the 0.5% solution of the substance. In the remaining 3 experiments all infected mice died like the controls. These findings seemed to suggest that, if at all, only a slight effect took place *in vitro.*

If unwashed mixtures of infective brain suspensions in concentrations ranging from 10^{-2} to 10^{-5} were prepared in 1% solution of Ro 2-3532/1 and injected into groups of 10 mice after 2 hours at room temperature (24°C), a survival rate of 70 to 100% was observed only with the 10^{-5} virus dilution. This represents a slightly greater anti-viral activity than is observed, if corresponding amounts of the compound and the virus were injected separately at the same site. However, the same result was obtained, if the mixture was injected immediately, indicating that prolonged exposure had failed to produce additional destruction of the virus.

Although the results *in vitro* were not entirely conclusive, there are, nevertheless, indications that Ro 2-3532/1 and related compounds possess direct anti-viral properties towards Col SK or MM virus at least *in vivo.* The most important is the fact that mice, surviving after administration of the active compound, could be successfully re-infected with a challenging dose (0.1 ml 10^{-5} dilution) of the virus 11-17 days after the infection. The reinfected animals succumbed to the infection at the same time as the controls.

Discussion. It is known that reproducible chemoprophylactic and chemotherapeutic effects in infections with small neurotropic viruses have only been rarely observed. We may add that 700 compounds from different chemical groups were tested in the Col SK and MM virus infections in this laboratory at the same time the naphthoquinonimines were studied and were found ineffective. Apart from the benzidine tetrazo dyestuffs(1) other substances such as arsenicals(5), certain sulfonamides(6), malononitrile(7) which had been described as active, were re-investigated but like other investigators(8-13) we were unable to confirm these results. The interesting observations by Verlinde and de Baan(14) on the effect of *V. cholerae* filtrates in the infection of mice with SK and MM virus which was confirmed by Jungeblut(15) could also be reproduced by us. It appeared, however, doubtful whether the active principle of the crude filtrates was actually identical with Burnet's receptor destroying enzyme (RDE).

The observations with the naphthoquinonimine Ro 2-3532/1 as described in the experimental part suggest that this substance is able to control the infection of mice with Col SK virus with a marked degree of consistency. Recent work by Jungeblut(16) confirms and extends our findings. Although the effect of the new compound was limited to the *intra-abdominal infection* and could only be demonstrated with moderately high infective doses of the virus and short intervals between infection and treatment, the type of anti-viral activity suggested some form of direct interaction of the active substance and the virus. This might be inferred by the efficacy of a single administration, by the relation of the dosage to the effect and by the activity of the substance, if injected remote from the site of the infection. The experiments also seem to indicate that once virus has entered the cells of the host, it can no longer be influenced by the compound. Therefore, it might be assumed that the 'free' virus is prevented by the compound from reaching the cells in which it can multiply. This seems to be also borne out by the lack of immunity of the surviving animals.

In the absence of convincing evidence that the naphthoquinonimine exerted a direct activity on the virus *in vitro*, it may yet be assumed that one of the breakdown products of the large molecule which are formed by the organisms of the host might be the active principle *in vivo*. Further work on this problem is in progress.

Summary. (1) The group of 2-hydroxy-1,4-naphthoquinonimines contains compounds which show activity against infections with Col SK and MM virus in mice. The effect of one member of this class of compounds, designated Ro 2-3532/1 is described in detail. (2) A single intra-abdominal injection of the new compound (500 mg/kg), administered shortly after an intra-abdominal infection with 10-20 LD_{50} of Col SK virus prevented the death of approximately 80% of the animals. A single subcutaneous injection of the substance under similar experimental conditions protected about 50% of the animals. Surviving mice were fully susceptible to reinfection with Col SK virus. (3) The effect of the intra-abdominal administration of Ro 2-3532/1 was dependent on the dose of the compound, on the time of treatment and on the infective dose of the virus. (4) Successful treatment by either the intra-abdominal or subcutaneous route was only observed in intra-abdominally infected mice. Infections induced by intracerebral, intramuscular, subcutaneous or plantar injections of the virus did not respond to the treatment with Ro 2-3532/1.

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Simplified Technic for Partial Hepatectomy in Rats with Fatty Livers.* (18719)

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In the course of experiments on rats on high fat diets, a technic for partial hepatectomy was developed which has not been previously described. This procedure, even when applied to rats on high fat diets, has resulted in a low operative mortality, minor post operative infections and has avoided the complication of the rat's chewing at the wound. In place of an abdominal incision as used by other investigators(1,2,3,4), the incision is made dorsally. Following the skin incision, an incision is made to the left of the spinal column along the lateral edge of the lumbar muscle. This incision is widened by expanding the scissors in the incision, and then extending the incision along the margin of the ribs for a short distance. A small, curved artery clamp is placed on the muscle at the upper corner of the incision and by pressing on the abdomen, the liver can be delivered through the incision. Using a piece of gauze, the left lobe of the liver is drawn out and then the median lobe. At this point, by exerting pressure on the clamp holding the upper corner of the wound, there is adequate space for placing a clamp around the two lobes of the liver. A coarse linen tie is placed behind the clamp and the lobes of the liver are excised. The remaining liver lobes are permitted to drop back into the abdomen. The muscle is closed with interrupted sutures and then the skin is closed. The operation requires no more than 10 minutes. There is a minimum of bleeding. The animals are unable to reach the wound and only minor post operative complications are encountered. The procedures are illustrated in Fig. 1.

TABLE I. % Composition of Diet.

	Diet C_2 %
Hydrogenated cotton seed oil	36
Cod liver oil	2
Casein	10
Sucrose	47
Salt mixture	5

Diet was supplemented as follows in mg/100 g diet: Thiamine 1 mg, pyridoxine 1 mg, riboflavin 2 mg, pteroylglutamic acid .2 mg, biotin .05 mg, Ca pantothenate 1 mg, niacin 10 mg, mixed tocopherols 15 mg, ''synkavit'' (2-methylnaphthohydroquinone 1,4-tetra-sodium diphosphate) 15 mg. Composition of salt mixture in %: ferric citrate 2.3, Ca diphosphate 44, K citrate 42, Mg citrate 11.6, Cu sulfate .08, K iodide .01%. Drinking water: 1% NaCl.

The diet used in these experiments is shown in Table I. This type of high fat and low protein diet results in fatty infiltration of the liver varying in degree from one animal to another. The weights of the individual liver lobes were determined after 62 days on the diet and, are shown in Table II. The weights of the lobes of the livers of normal male rats from our rat colony (Long-Evans strain) are also given for comparison. The weights of the liver lobes of normal rats, as % of total liver weight, agree closely with values reported by other observers(2,4), although liver weight as % of body weight is somewhat greater in our rats. In spite of the increase in the weight of the liver lobes, due to fatty infiltration, the relationship of the lobes to one another did not differ from that of normal rats. The liver removed by partial hepatectomy in our experiments therefore represents 71.3 ± 2.9% of the total liver. In an occasional rat, the median lobe consisted of 3 rather than 2 parts, and when this occurred the weight of the median lobe amounted to about 50% of the total weight of the liver.

The hepatectomies were done on male rats that had been on a high fat diet for 42 to 62

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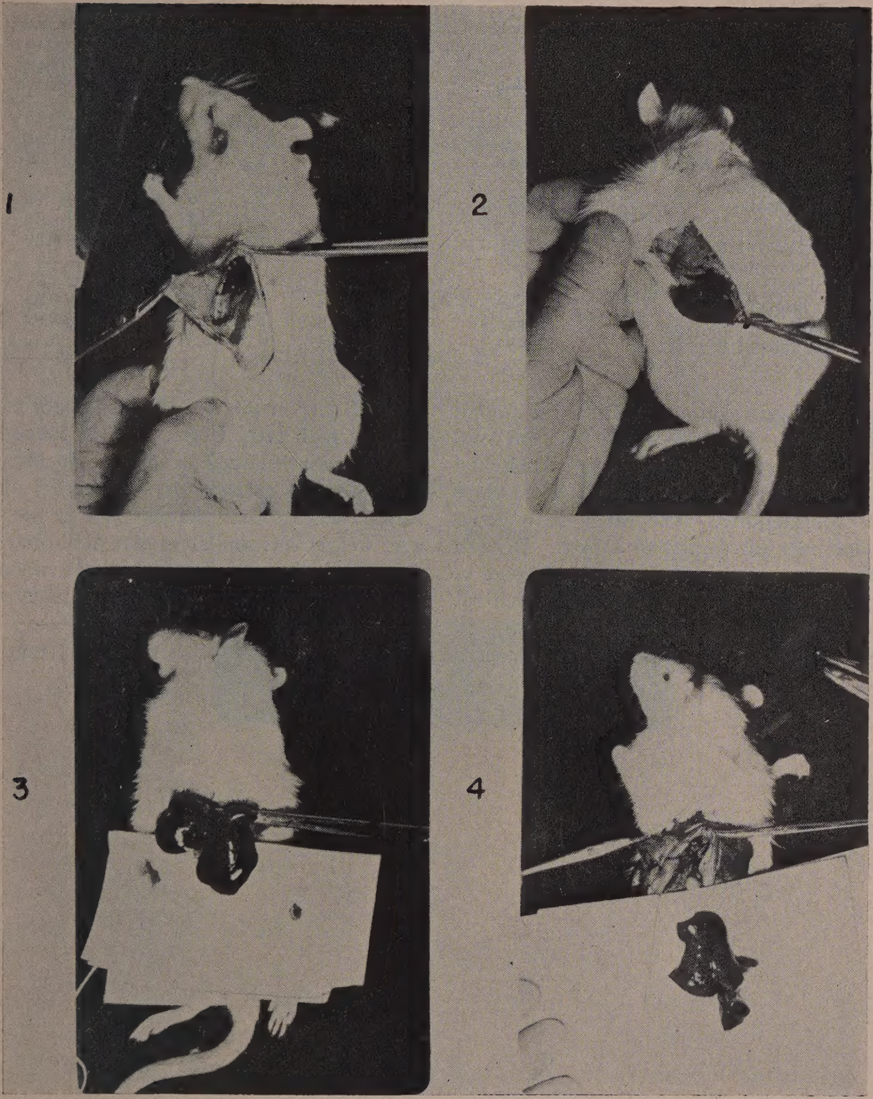

FIG. 1
Technic for partial hepatectomy in the rat.

days, which meant that the liver, at operation, was fatty, large, and more readily traumatized than the normal liver. In spite of this, the immediate operative mortality was fairly low, averaging 24% of a total of 89 rats during the first year of these experiments and 4% of a total of 25 rats during the second year. Post-operative care consisted of the administration of aqueous penicillin and 20% glucose in 1% NaCl as drinking water during the first 24 hours. Other observers(2,3) have reported a surgical mortality of about 5% following partial hepatectomies by the abdominal route in rats on normal diets, while rats on a 13% fat diet were reported *not* to survive this operation(3).

This procedure has enabled us to obtain chemical data on the liver of a rat at the time

TABLE II. Relation of Weight of Liver Lobes to Total Liver Weight.

	10 normal rats		10 rats on high fat diet	
	Range	Mean Stand. Error	Range	Mean Stand. Error
Wt of rats—g	150-250	200 ± 3.02	118-207	170 ± 8.20
Wt of liver—g	6.38-9.53	7.77 ± .30	6.32-15.86	9.63 ± .93
Liver wt as % of body wt	3.08-4.61	3.92 ± .16	3.31-9.60	5.76 ± .59
Wt of liver lobes—g				
Left lateral	1.84-3.35	2.61 ± 1.58	2.44-5.60	3.31 ± .29
Median	2.22-3.34	2.76 ± .13	2.00-7.16	3.58 ± .48
Right lateral & caudate	1.20-3.10	2.40 ± .16	1.55-4.92	2.74 ± .30
Wt of liver lobes as % of total liver wt				
Left lateral	26.2-39.5	33.45 ± 1.19	28.8-38.6	34.73 ± 0.83
Median	32.0-52.3	35.77 ± 1.98	31.6-54.0	36.61 ± 2.09
Right lateral & caudate	18.9-34.8	30.77 ± 1.54	17.2-33.5	28.66 ± 1.59

of operation, and again at the time the animal was sacrificed. We have found that the total lipid content of the liver of rats which had been on the high fat diet for 42 to 62 days varied considerably. The range in a series of 41 animals was from 4 to 23 g%. In view of the wide variation in the lipid content of the liver, the opportunity of having each rat act as its own control is advantageous.

Summary. A method is described for partial hepatectomy in the rat which is applicable to animals with fatty livers. It was found that in rats with enlarged livers, due to fatty infiltration, the relation of the weights of the various lobes to one another and to the total liver weight was similar to that in normal rats. Apparently, the individual lobes maintain their relationship in spite of fatty infiltration.

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